UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Departamento de Enxeñaría Química

Valorisation of saline wastewaters: a challenge for the obtainment of bioproducts

Memoria presentada por

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Para optar ao grado de Doutor pola
Universidade de Santiago de Compostela

Santiago de Compostela, Maio de 2017

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List of Acronyms

AD	Anaerobic Digestion
ADF	Aerobic dynamic feeding
AEBR	Anaerobic Expanded Bed Reactor
AGS	Aerobic Granular Sludge
ANFACO	Asociación Nacional de Fabricantes de Conservas de Pescado
APHA	American Public Health Association
ASBR	Anaerobic Sequencing Batch Reactor
ATU	Allyltiourea
AWWA	American Water Works Association
BD	Biodegradability
BMP	Biomethane Potential
BOD	Biological oxygen demand
BSA	Bovine Serum Albumine
C	Carbon
CH	Carbohydrate
COD	Chemical oxygen demand
COD_S	Soluble chemical oxygen demand
COD_T	Total chemical oxygen demand
CSTR	Continuous Stirred Tank Reactor
Cy3	Cyanine 3
DAIME	Digital Image Analysis in Microbial Ecology
DAPI	4',6-DiAmidino-2-Phenylindole
DNA	Deoxyribo-Nucleic Acid
DO	Dissolved oxygen
DS	Dissolved sulphide
DSC	Differential scanning calorimetry
EDTA	Ethylene-Diamine-Tetra-Acetic acid
EASME	Executive Agency for Small and Medium Sized Enterprises
F	Formamide
F/M	Food to microorganism ratio
FA	Free ammonia
FAS	Ferrous Ammonium Sulphate
FBR	Fluidized Bed Reactor
FID	Flame ionization detector
FISH	Fluorescent *In Situ* Hybridization
FITC	Fluorescein IsoThioCyanate
FLAS	Flocculent Activated Sludge
FS	Free sulphide
GC	Gas Cromatography

GC-MS	Mass spectrometry
HB	Hydroxybutyrate
HV	Hydroxyvalerate
HRT	Hydraulic Retention Time
HAc	Acetic acid
HPr	Propionic acid
HBu	Butyric acid
HVA	Valeric acid
IA	Intermediate alkalinity
IC	Ion chromatography
IC_{50}	Half Maximal Inhibitory Concentration
MMC	Microbial mixed culture
M_n	Number average molecular weight
MP	Methane Production
MPM	Methane Producing Microorganisms
MSA	Maximum specific activity
M_w	Average molecular weight
N	Nitrogen
OLR	Organic Loading Rate
P3HB	Poly(3-hydroxybutyrate)
P3HV	Poly(3-hydroxyvalerate)
P4HB	Poly(4-hydroxybutyrate)
P4HV	Poly(4-hydroxyvalerate)
P(3HB-co-3HV)	Copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HB-co-4HB)	Copolymer poly(3-hydroxybutyrate-co-4-hydroxybutyrate)
PA	Partial alkalinity
PBS	Phosphate Buffer Solution
PDI	Polydispersity index
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHBV	Copolymer polyhydroxybutyrate-co-hydroxyvalerate
PHV	Polyhydroxyvalerate
PP	Polypropylene
PS	Polystyrene
q	Rate
RWC	Reactor treating the washing of the tuna boilers
RWW	Reactor treating the effluent of the tuna boilers
S	Sulphur
SBR	Sequencing Batch Reactor
SEC	Size exclusion chromatography
SMA	Specific Methanogenic Activity

SRB	Sulphate Reducing Bacteria
SRT	Solids Retention Time
T	Temperature
TA	Total alkalinity
TCA	Tricarboxylic acid (cycle)
TCD	Thermal conductivity Detector
T_d	Decomposition temperature
T_g	Glass transition temperature
T_m	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
TSS	Total Suspended Solids
TS	Total solids
UASB	Upflow Anaerobic Sludge Blanket
UK	United Kingdom
US	United States
USA	United States
UV	Ultraviolet
VFA	Volatile Fatty Acid
vol%	Percentage in volume
V	Volatile solids
VSS	Volatile suspended solids
WC	Wastewater from tuna boilers
wt%	Percentage in weight
WW	Wastewater from the washing of the tuna boilers
WWTP	Wastewater Treatment Plant
X	Biomass
Y	Yield
ΔHm	Melting enthalpy

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Resumo

A presente tese propón a obtención de produtos de alto valor engadido a partires de refugallos da industria con alto contido en sal.

Os produtos de alto valor engadido contemplados na presente tese son: (1) O biogás é coñecido por ser unha enerxía renovable producido a través da dixestión anaerobia. (2) Os ácidos graxos obtéñense tamén da dixestión anaerobia de refugallos pero sen permitir ao proceso continuar ata a etapa de metanoxénese. A partires de estes compostos pódense sintetizar substancias químicas, obter biocombustibles ou mesmo bioplásticos. (3) Os polihidroxialcanoatos obtéñense a partir do tratamento aerobio do efluente da acidificación anaerobia. Estes biopolímeros son coñecidos por teren propiedades semellantes ás dos termoplásticos convencionais e seren así candidatos a substituílos en certos ámbitos.

As industrias coas que se traballa de acuicultura así coma conserveiras de túnidos. Os vertidos industriais destas industrias semellan ser bos candidatos para o seu uso coma materias primas para a obtención de produtos de alto valor engadido sería beneficioso tamén xa que se reduce o volume de refugallos a tratar pola empresa. Os procesos propostos pretenden ser viables economicamente así coma respectuoso có medio ambiente en comparación coas enerxías e materiais derivados do cru.

O sistema de tratamento de efluentes salinos industriais propostos inclúe o arranque dos diferentes reactores así coma o seguimento da operación e caracterización dos influentes, efluentes e produtos de valor engadido. De feito, é coñecido que a operación dos reactores anaerobios vese afectado por diversos parámetros coma o pH, a temperatura, o tempo de retención, etc, polo que se revisará o efecto da dixestión anaerobia dun lodo salobre.

A produción de ácidos por medio da fermentación anaerobia tamén se ve afectada polos parámetros de operación, tanto en composición coma en rendemento da reacción, polo que tamén se investigan estes efectos durante a operación.

Por último, a parte máis novidosa da presente investigación radica na produción de biopolímeros. Os polihidroxialcanoatos son producidos en reactores aerobios empregando como substrato os ácidos graxos producidos na fermentación anaerobia dos refugallos industriais salinos de conserveira. Neste caso, de novo os parámetros operacionais coma a composición do substrato, pH, etc. inflúen no rendemento da operación así coma na composición e propiedades dos polímeros, de aí o interese en estudalos.

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Resumen

La presente tesis propone la obtención de productos de alto valor añadido a partir de residuos de la industria con alto contenido en sal.

Los productos de alto valor añadido contemplados en la presente tesis son: (1) El biogás, es conocido por ser una energía renovable producida a través de la digestión anaerobia. (2) Los ácidos grasos se obtienen también de la digestión anaerobia de residuos, pero sin permitir al proceso continuar hasta la metanogénesis. A partir de estos compuestos se pueden sintetizar sustancias químicas, biocombustibles o incluso bioplásticos. (3) Los polihidroxialcanoatos se obtienen a partir del tratamiento aerobio del efluente de la acidificación anaerobia. Estos biopolímeros son conocidos por tener propiedades semejantes a las de los termoplásticos convencionales y ser así candidatos a sustituirlos en ciertos ámbitos.

Las industrias con las que se propone trabajar son acuicultura y conserveras de túnidos. Estos vertidos industriales parecen ser buenos candidatos para la producción de biocombustibles y biopolímeros además de que su uso como materias primas para la obtención de productos de alto valor añadido sería beneficioso pues se reduce el volumen de residuos a tratar por la empresa. Los procesos propuestos pretenden ser viables económicamente, así como respetuosos que el medio ambiente en comparación con las energías y materiales derivados del crudo.

El sistema de tratamiento de efluentes salinos industriales propuestos incluye el arranque de los diferentes reactores, así como el seguimiento de la operación y caracterización de los influentes, efluentes y productos de valor añadido. De hecho, es conocido que la operación de los reactores anaerobios se ve afectado por diversos parámetros como el pH, la temperatura, el tiempo de retención, etc, por lo que se revisará el efecto de la digestión anaerobia de un lodo salobre.

La producción de ácidos también se ve afectada por los parámetros de operación, tanto en composición como en rendimiento de la reacción, por lo que también se investigan estos efectos durante la operación.

Por último, la parte más novedosa de la presente investigación radica en la producción de biopolímeros. Los polihidroxialcanoatos son producidos en reactores aerobios empleando como sustrato los ácidos grasos producidos en la fermentación anaerobia. En este caso, de nuevo los parámetros operacionales como la composición del sustrato, pH, etc. influyen en el rendimiento de la operación, así como en la composición y propiedades de los polímeros, de ahí el interés en estudiarlos

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Abstract

The present thesis proposes the obtainment of high value-added products from industrial waste with high content of salt.

The value-added products considered in the present thesis are: (1) Biogas, well-known of being a renewable energy produced by means of anaerobic digestion. (2) The fatty acids are also obtained by anaerobic digestion, but without allowing the process to continue to the methanogenesis stage. From these compound, chemicals, biofuels, or even bioplastics can be obtained. (3) Polyhydroxyalkanoates are obtained from the aerobic treatment of the acidified effluent from the anaerobic fermenter. These biopolymers are known to have comparable properties to conventional thermoplastics and are good candidates to substitute them in some fields.

The industries in this proposed work are aquaculture and tuna-canning ones. The industrial effluents of these sectors seem to be good candidates for the production of biofuels and biopolymers. This would be beneficial since it reduces the volume of waste that needs to be treated by the industry. The proposed processes are expected to be economically feasible and environmentally friendly in comparison with fuels and materials derived from crude.

The proposed system for the treatment of industrial saline effluents includes the start-up of the different reactors, the follow-up of the operation and the characterisation of the influents, effluents and value added products. In fact, it is known that the operation of the anaerobic reactors seems to be affected by diverse parameters like the pH, the temperature, the retention time, etc, and the effect of these parameters will be studied over the anaerobic digestion of saline sludge.

The production of acids by means of the anaerobic fermentation also seems to be affected by the operational parameters, but also their composition and yield, and these effects will be monitored and studied during the operation.

Finally, the novel part of the present investigation lies in the biopolymer production process. Polyhydroxyalkanoates are produced in aerobic reactors using fatty acids as substrates. These acids are produced in the anaerobic fermentation of the saline fish-canning wastewaters. In this case, the operational parameters like the composition of the substrate, pH, etc. influence again the performance of the operation. These parameters also influence the composition and properties of the biopolymers, so it is of interest studying them.

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Resumo estendido

O principal obxectivo da presente tese é a obtención de produtos de alto valor engadido a partires de augas residuais industriais con alto contido en auga de mar empregando cultivos mixtos de microorganismos.

Historicamente, os refugallos e residuos eran desbotados na natureza, creando problemas hixiénicos e sanitarios así coma medioambientais pero ata principios do século XIX non se empregaron colectores de augas residuais. Debido ao rápido crecemento industrial, a evolución do tratamento de augas durante o século XX foi inmenso. O tratamento biotecnolóxico convencional de augas baséase na eliminación de patóxenos, nutrientes e materia orgánica para cumprir coa normativa vixente mais este sector está actualmente a sufrir unha evolución cara o desenvolvemento de tecnoloxías limpas que permitan obter produtos de alto valor engadido en vez de só eliminar contaminantes. Isto implica que a auga residual é considerada un recurso ou materia prima, permitindo diminuír o impacto ambiental e reducir os consumos enerxéticos. En relación a isto, a Unión Europea está a promover un novo modelo económico baseado nun sistema rexenerativo chamado "Economía Circular" para así substituír o actual modelo baseado na fabricación-consumo-eliminación. Este novo modelo pretende optimiza-los recursos e minimiza-los riscos ademais de incentivar a mellora da economía europea. Como se pode observar, o emprego de augas residuais como materia prima e a recuperación de recursos a partires destes residuos son puntos clave para a estratexia europea sobre crecemento sustentable.

Seguindo coa idea de economía circular e crecemento sustentable, os investigadores nos últimos anos dedicáronse de xeito importante á valorización de residuos, especialmente no campo das augas residuais. Os principais produtos estudiados son o biogás, os ácidos orgánicos, os alcohois e os biopolímeros e poden ser obtidos a partires de residuos coma lodos de depuradora, augas residuais industriais, residuos do sector primario, etc.

Seguindo nesta liña, a presente tese pretende obter biogás, ácidos graxos volátiles (AGV) e polihidroxialcanoatos (PHA) a partires de augas residuais de industrias do sector da acuicultura e do sector conserveiro de produtos mariños. O motivo de escoller este tipo de efluentes é o seu alto contido en materia orgánica ademais de que

estes dous sectores teñen ampla presenza en Galicia, xerando elevados volumes de augas residuais. Por exemplo, o 87 % en volume da produción de conservas de peixe españolas ten orixe galego. Estas augas residuais son tratadas convencionalmente mediante métodos físico-químicos (coagulación-floculación, filtración, etc), que soen estar caracterizados polo seu elevado custo, xa que hai que contar co gasto en reactivos pero tamén có gasto que supón a xestión dos lodos xerados. Unha alternativa a estes procesos é o emprego de sistemas biolóxicos para o seu tratamento, coa vantaxe de obter produtos de alto valor engadido.

Neste senso, a presente tese pode ser dividida en tres bloques: un primeiro sobre a produción de biogás a partires de lodos salinos (**Capítulo 3**), un segundo bloque sobre a produción de AGV a partires de dous efluentes distintos de conserveira de atún (**Capítulo 4**), e o terceiro e meirande bloque sobre a produción de PHA (**Capítulos 5, 6, 7 e 8**) empregando AGV como substrato e estudando a influenza do sal na acumulación e propiedades dos mesmos. Ademais, no **Capítulo 1** preséntase unha introdución a cada un dos temas propostos (biogás, AGV e PHA) así coma unha revisión do estado actual de cada unha das tecnoloxías. No **Capítulo 2** descríbense os materiais e métodos empregados na presente tese.

Os contidos principais de cada un dos capítulos así coma os obxectivos acadados son descritos a continuación:

Na primeira parte do **Capítulo 1** ofrécese unha visión xeral sobre os procesos convencionais para a obtención de biogás así coma un resumo das condicións que afectan ao proceso, xa que está reportado que a presencia de NaCl afecta a eficiencia da dixestión anaerobia, resultando un contratempo para o seu tratamento. A presencia de sulfuro tamén pode supoñer un problema para o tratamento anaerobio. Na segunda parte deste capítulo atópanse os métodos para a obtención de AGV empregando augas residuais de conserveira como substrato e, de novo, o proceso se atopa baixo condicións de alta salinidade, afectando ao sistema anaerobio pero tamén á composición dos ácidos. Finalmente, a terceira parte corresponde ao groso da introdución, e vai dedicado á produción de PHA en cultivo mixto xa que é o ámbito máis novidoso e no que máis se traballou nesta tese. Trátanse temas dende a configuración do sistema para a produción de biopolímeros ata as propiedades dos PHA como termoplásticos.

No **Capítulo 2** descríbense con detalle os materiais e métodos empregados na presente tese. Estas análises engloban as fases gasosa, líquida e sólida. Os métodos descritos no *Standard Methods for the Examination of Water and Wastewater* foron empregados para a determinación de certos parámetros convencionais como a demanda química de osíxeno (DQO), a alcalinidade ou a concentración de sólidos en suspensión. Outras análises foron adaptadas doutra bibliografía para a análise de proteínas ou PHA. Métodos máis avanzados foron empregados para a determinación das propiedades dos biopolímeros. Tamén se describe a aplicación da técnica de hibridación *in situ* con fluorescencia (FISH) para a identificación de poboacións microbiolóxicas así como do programa DAIME para a cuantificación aproximada de devanditas poboacións. Finalmente preséntase con detalle os cálculos empregados na tese para a determinación da cantidade de biogás producida, ou dos rendementos e velocidades de produción de PHA, entre outros.

No **Capítulo 3** estudouse o arranque e operación dun reactor anaerobio para a produción de biogás empregando dous substratos diferentes (lodo granular aerobio e lodo floculento aerobio) e baixo diferentes concentracións de sodio (2.1 – 6.7 g Na^+/L). O obxectivo deste capítulo é estudar e avaliar o comportamento dun dixestor anaerobio tratando dous lodos distintos e comprobar se a degradación anaerobia de lodo granular é comparable é degradación de lodo floculento. Ademais, outro obxectivo é estudar o potencial para producir biogás de ámbolos dous lodos baixo diferentes condicións de salinidade. A presenza de sulfuro nas fases líquida e gasosa foi revisada debido a súa posible influenza sobre o proceso. Para isto empregouse un reactor de escala laboratorio de 5 L a 35 °C e axitado continuamente a 100 rpm. O reactor operou cun tempo de retención hidráulica (TRH) de 20 días. Nunha primeira etapa o reactor tratou o lodo aerobio granular con cargas orgánicas de 0.4 - 1.6 g DQO/(L · d) e baixo diferentes concentracións de salinidade (2.1 – 6.7 g Na^+/L e 0.5 – 1.5 g SO_4^{2-}/L). Nunha segunda etapa o reactor tratou o lodo floculento cunha carga orgánica de 1.5 g DQO/(L · d) e concentracións de sales de 2.6 g Na^+/L e 0.7 g SO_4^{2-}/L. Os valores obtidos para a biodegradabilidade de ámbolos dous lodos foi do 32% para o lodo granular e do 27% para o floculento, o que indica que o estado de agregación non limitou o proceso. A auga de mar contida nos lodos levou a concentracións de sulfuro dentro do reactor de 38 - 93 mg/L e de 1.5 – 3.8% no biogás, indicando que este debe ser pre-tratado no caso de querer empregalo para a produción de enerxía.

No **Capítulo 4** estúdase a capacidade de produción de AGV empregando dúas augas residuais distintas procedentes da industria conserveira: unha delas procedente de lavar os cocedoiros de atún e outra delas sendo a propia auga de cocción do atún. Ámbalas dúas augas tiñan un contido en materia orgánica moi diferente, sendo aproximadamente 4.16 g DQO_S/L para a auga de lavado e de 16.3 g DQO_S/L para a de cocción. O obxectivo é estudiar o comportamento dos reactores así coma a capacidade de produción de AGV e a súa composición en función do substrato empregado. Para auga de baixa carga empregouse un reactor secuencial descontinuo de 1.8 L a 150 rpm e 30 °C que operou en ciclos de 12 h cun TRH de 1 día. A salinidade neste reactor foi de 0.95 ± 0.07 g NaCl/L. Para tratar a auga de cocción empregouse un reactor semi-continuo de 1.5 L a 150 rpm e 35 °C que operou cun TRH de 2 días, cunha concentración dentro do reactor de 22.4 ± 4.3 g NaCl/L. A eficiencia dos reactores, medida como a cantidade de DQO inicial que pasou a AGV, foi do 63% para auga de lavado e do 87% para a auga de cocción. En ámbolos dous casos, o ácido acético foi o producido maioritariamente, con valores de 45.5 DQO_{AGV}% e 43.1 DQO_{AGV}% para a auga de lavado e a de cocción, respectivamente. A meirande diferenza foi atopada no feito de que o segundo ácido máis producido foi o propiónico (27.7 DQO_{AGV}%) no caso das augas de lavado e butírico (38.4 DQO_{AGV}%) no caso das augas de cocción. Está reportado por outros autores que a produción de butírico se ve favorecida pola presenza de altas concentracións de sal, neste caso de 22 g NaCl/L aproximadamente. A pesares das altas porcentaxes de acidificación, estes efluentes non son axeitados para a produción de enerxía debido á salinidade e tamén ás altas concentracións de amonio. Unha opción alternativa sería empregalos para a obtención de PHA.

No **Capítulo 5** estúdase o arranque e comportamento dun reactor para o enriquecemento dun cultivo mixto así coma a capacidade de acumulación do mesmo. Esta capacidade de acumulación é comprobada tanto para o substrato empregado no enriquecemento, como é habitual neste tipo de sistemas, como con outros substratos, para así comprobar a versatilidade do sistema e poder deseñar distintos biopolímeros sen a necesidade de arrancar un novo sistema de enriquecemento para cada tipo de substrato. O obxectivo deste estudo é avaliar a acumulación de biopolímero empregando distintos substratos, estudar o efecto producido polo cambio de substrato sobre os parámetros cinéticos e estequiométricos, e, finalmente, estudar a influencia da relación alimento – microorganismo (relación F/M) sobre a acumulación. O reactor de enriquecemento é un reactor secuencial de 1.8 L completamente aireado e axitado, que operou a 30 °C en ciclos de 12 horas cun TRH de 1 día. Para o

enriquecemento empregouse unha mestura de AGV como substrato de composición 1.52/0.61/0.15/0.25 g/L de ácidos acético (HAc), propiónico (HPr), butírico (HBu) e valérico (HVa), respectivamente. O reactor de acumulación operou por lotes como reactor descontinuo, baixo as mesmas condicións de operación que o reactor de enriquecemento, cós seguintes substratos: a mestura de AGV empregada no enriquecemento, HAc, HPr, HBu e HVa. O substrato foi suplementado en pulsos. O resultado destas acumulacións levou á obtención de biopolímeros de composicións moi diferentes, que variaban o seu contido en hidroxivalerato (HV) desde o 2 ata o 76 %peso do contido total en PHA mentres que o máximo contido en PHA oscilou entre 39.0 – 48.4 %peso para tódolos ensaios. Os rendementos das acumulacións foron moi elevados, con valores de 0.683 - 0.838 Cmol PHA/Cmol AGV para tódolos ensaios. Estes rendementos vense afectados pola relación F/M, cuxo óptimo foi atopado no rango 1 – 7 Cmol AGV/(Cmol X · ciclo) xa que valores maiores de 7 levan a inhibición por substrato. Como se pode observar á vista dos resultados, a produción de PHA a partir de substratos diferentes aos do enriquecemento é posible.

No **Capítulo 6** explórase a posibilidade de producir PHA cando hai presencia puntual de NaCl. Este estudo é preciso no caso de querer empregar como substrato efluentes con alto contido en sal, coma por exemplo os do Capítulo 4. Para isto investigouse a influencia da salinidade na acumulación por medio de ensaios que empregaron concentracións de sal de 0, 7, 13 e 20 g NaCl/L. Empregouse mesmo cultivo enriquecido do capítulo anterior. Os ensaios de acumulación foron feitos en pulsos coa mestura de AGV empregada para alimentar o reactor de enriquecemento (1.52/0.61/0.15/0.25 g/L de HAc, HPr, HBu e HVa, respectivamente) a 30 °C e completamente aireado e axitado. A acumulación de PHA sen presenza de NaCl foi do 53 %peso mentres que a 7, 13 e 20 g NaCl/L este valor foi de 35, 17 e 9 %peso, respectivamente. A composición en HV variou entre 27 e 14 %Cmol, diminuíndo a medida que aumentaba a salinidade. Isto podería ser explicado polo feito que a maior concentración de sal e maior inhibición o HPr é empregado para mantemento celular con preferencia sobre o HAc, obtendo así máis polihidroxibutirato (HB) acumulado ca HV. De feito, os rendementos de HB mantivéronse practicamente constantes con independencia da concentración de NaCl, cun valor medio de 0.52 Cmol HB/Cmol AGV, mentres que os de HV diminuíron desde 0.19 ata 0.06 Cmol HV/Cmol AGV a medida que a concentración de sal aumentou nos seguintes ensaios descontinuos. De tódolos xeitos, os parámetros cinéticos víronse afectados sempre pola presencia de sal no medio.

No **Capítulo 5** estudiábase a posibilidade de producir PHA con diferentes substratos na acumulación partindo dun mesmo enriquecemento. É coñecido que o substrato inflúe na composición e propiedades dos PHA obtidos, aínda que a día de hoxe é difícil predicir o biopolímero que se vai obter cando se traballa con cultivos mixtos. Por outra banda, no **Capítulo 6** estudouse a influencia do NaCl sobre a acumulación e tamén se viu que afectaba á composición do biopolímero. Agora, no **Capítulo 7**, estúdase máis en profundidade como afecta a variación de substrato na acumulación e a presenza de NaCl no medio ás propiedades do polímero, en termos de peso molecular e propiedades térmicas. Ademais, tamén se estuda como afecta a etapa de purificación a devanditas propiedades por medio de testar dous métodos de extracción diferentes. A relevancia deste estudo radica na pouca información que hai con respecto a propiedades de PHA así como a nula información sobre a influencia de inhibidores (coma o NaCl) no medio, que poden influír nas propiedades poliméricas do bioplástico. As acumulacións realizáronse coma sempre nun reactor descontinuo a 30 °C completamente axitado e aireado. Os substratos empregados foron HAc, HPr e a mestura de ácidos do enriquecemento (ver **Capítulo 5**). Para poder determinar os valores dos parámetros relacionados có peso molecular empregouse a cromatografía de exclusión por tamaño (SEC en inglés), que deu valores de peso molecular medio (M_w), número medio do peso molecular (M_n) e do índice de polidispersión. Os valores das propiedades térmicas foron determinados por calorimetría diferencial de varredura (DSC en inglés). Os valores obtidos foron de temperatura de transición vítrea (T_g), temperatura de fusión (T_m) e temperatura de degradación (T_g). Os método de extracción empregado foi o do cloroformo mentres que o método de purificación foi por precipitación con etanol xeado. As impurezas foron detectadas por cromatografía de masas. Os polímeros obtidos tiñan un contido en HV que variou do 4 %peso de PHA ata o 66 %peso de PHA. As propiedades térmicas amosaron unha clara dependencia coa composición do polímero agás T_d, que se mantivo practicamente constante. Pódese ver que a proporción de HV é un bo indicador das propiedades do polímero e como afecta tanto ao peso molecular como ás temperaturas de transición vítrea e de fusión. A salinidade afectou á composición dos biopolímeros acumulados e, polo tanto, afectou ás propiedades dos mesmos. A variable máis afectada foi o peso molecular que pasou de 1.2 x 10^5 g/mol a 2.0 x 10^5 g/mol. Isto aconteceu debido probablemente a unha redución das cadeas de polímero máis curtas debido á inhibición que produciu a presencia de NaCl. A purificación non afectou ao peso molecular mais si afectou á cristalinidade, que se viu incrementada en máis dun 70 %, e tamén afectou ás propiedades térmicas, xa que se viron incrementadas, por exemplo,

de -21.0 °C ata -11.0 °C no caso de T_g e de 91.7 °C ata 104.6 °C no caso de T_m para biopolímeros producidos con HPr Estes valores foron de -6.6 °C ata 5.3 °C no caso de T_g e de 161.4 °C ata 165.2 °C no caso de T_m para biopolímeros producidos con HAc. Ante os resultados observados, pódese afirmar que a presencia de NaCl promove a acumulación de HB sobre a de HV así coma un polímero cun maior peso molecular; ademais, a purificación afecta ás variables térmicas sobre todo, pero só debería ser realizada no caso de que o posterior uso do biopolímero requira unha alta pureza, xa que os custes da purificación aumentarían o custe do polímero e limitaría a súa comercialización.

No **Capítulo 8** estúdiase a posibilidade de obter PHA con cultivo mixto a partir de ácidos orgánicos obtidos a partir de augas de conserveira fermentadas. Diferentes residuos fermentados xa foron probados para producir PHA (residuos da industria do aceite de oliva ou da caña azucreira, soro de leite, lodos activos, etc), mais todos eles diferéncianse da auga de conserveira por careceren de cantidades elevadas de proteínas e en NaCl. No **Capítulo 6** desta tese estudouse o efecto do sal sobre a capacidade de acumulación do cultivo microbiano mixto e no **Capítulo 7** o seu efecto sobre as propiedades do polímero. Cabe esperar que a presencia de NaCl afecte ao sistema, así coma o alto contido en amonio debido á presencia de proteínas na auga residual. Os obxectivos principais deste capítulo é estudiar o enriquecemento con augas fermentadas de conserveira, cuxo proceso de acidificación estúdase no **Capítulo 4**. O reactor de enriquecemento é un reactor secuencial de 1.8 L completamente aireado e axitado, que operou a 30 °C en ciclos de 12 horas cun TRH de 1 día. A composición alimentada é de aproximadamente 28.8:16.4:47.8:6.1 DQO% de HAc:HPr:HBu:HVa, respectivamente, ademais dunhas concentracións de amonio de 228.9 ± 47.7 mg NH4+-N/L e unha salinidade de 3.11 ± 0.80 g NaCl/L. O reactor de acumulación estaba completamente aireado e axitado, e operou a 30 °C. A capacidade de acumulación avalíase por medio de dúas estratexias diferentes de alimentación: unha en pulsos e outra nun único pulso inicial. Os mellores resultados en termos de acumulación foron acadados polo reactor en pulsos, que acadou preto dun 9 %peso de PHA, mentres que a produtividade resultou ser mellor para o ensaio onde se alimentou cun só pulso (55.4 mg PHA/(L · h)) en comparación con varios pulsos (10.31 mg PHA/(L · h)). A presenza de amonio favoreceu o crecemento da biomasa en ámbolos dous ensaios, con valores de 0.366 e 0.266 Cmol X/Cmol VFA para o ensaio por pulsos en un único pulso, respectivamente, aínda que se pode observar que o rendemento da biomasa foi menor para o caso dun único pulso. Semella que alimentar cun único pulso

diminúe o crecemento a prol da produtividade, aínda que máis ensaios precisan ser feitos.

Chapter 1
INTRODUCTION

SUMMARY

The present chapter describes the scope of this thesis.

The first part of this document describes a conventional method for the removal of the organic matter together with the recovery of energy as biogas when treating saline wastewaters. Another relevant point of this thesis is the obtainment of volatile fatty acids (VFAs) by means of the fermentation of a fish-canning wastewater, which has a relevant concentration of sodium chloride. Nevertheless, the gross of the research work is dedicated to the obtainment of biopolymers with an enriched microbial mixed culture using VFAs as substrate. This is the most novel and innovating part of the performed research taking into account that the assayed substrate contained relevant concentrations of sodium chloride.

Among the main investigations of this thesis were: the influence of the presence of sodium chloride, the impact of operational conditions -like solids or hydraulic retention time, pH, ammonia concentration, etc- and the promotion of more specific bacterial populations so as to obtain different value added products from saline wastewaters. Bibliographic references were used for comparison of what has been done up to date to justify the novelty of the different parts of this thesis.

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1.1 RESOURCE RECOVERY

Historically, wastewater treatment evolved from the situation where just getting rid of the residue was the objective to focus on its treatment and management. For centuries, the most widespread practice was to discard the residue, using the environment as the receptor. This practice generated significant sanitary and hygienic problems. Ancient Greeks designed sewer systems to carry the wastewaters far from the city centres and the Romans improved these constructions. Nevertheless, all these advances were forgotten when the Empire fell down. Sewers were not recovered until the beginning of the XIX century (Wiesmann et al., 2007).

At the dawn of the XX Century the pressing need for effective wastewater treatments was due to the increasing population and the industrial progress. Until this date, even the river where the wastewaters were discharged was considered as part of the treatment process (Wiesmann et al., 2007). Nevertheless, in the mid-XX century the problem of eutrophication arose and the need for eliminating nitrogen and phosphorous arose. By this time, advances in aerobic and anaerobic wastewater treatment systems developed in parallel. The traditional biotechnological wastewater treatments were mainly focused on the elimination of contaminants and nutrients by generating a liquid flow which could be disposed in the environment without any harm (Kleerebezem and van Loosdrecht, 2007). These types of biological treatments were mainly focused on (Tchobanoglous et al., 2003):

- Removal of colloidal, suspension and floating particles
- Treatment of the biodegradable organic matter
- Removal of pathogens

At the end of the XX Century, the evolution of wastewater treatment plants was patent by the development of new processes and technologies like the up-flow anaerobic sludge blanket, anammox-based processes, etc. However, the most important fact was the new concept of developing technologies to treat the wastewater to obtain value-added products instead of just to remove pollutants (Kleerebezem and van Loosdrecht, 2007). Nowadays, the trend is to design new processes without any environmental impact. This means that the energy consumption must be optimised and reduced; the residue should be seen as a resource; the environmental footprint has to be reduced; and, of course, the pollutants must be removed to guarantee the right water quality for discharge.

1.1.1 Circular economy

The current economic model is based on the fabrication, use and disposal of the material and even energy. The new model is based on a regenerative and recovering design which is called "*Circular Economy*". It is a continuous loop about production, use, and recycling (Figure 1.1) which has the aim of optimising the resources and minimise the risks. The European Commission intends to encourage the transition towards the circular economy since it will boost the economy (European Commission-Environment, 2016d).

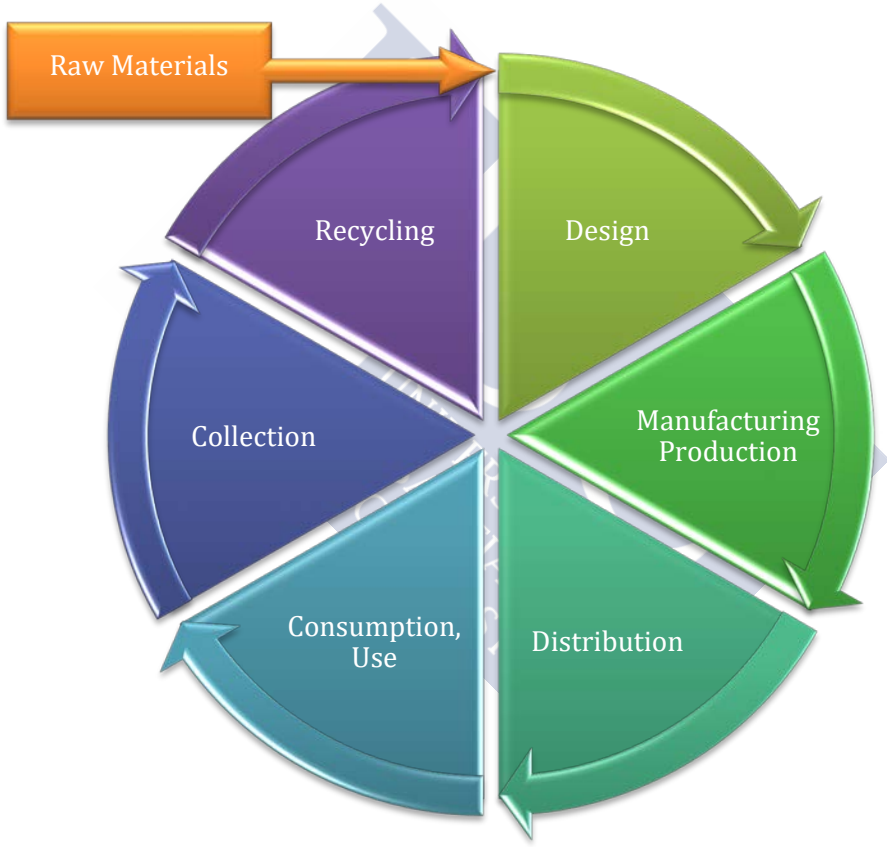


Figure 1.1. Circular economy design proposed by the European Commission (European Commission-Environment, 2016a).

According to data collected from the European Commission of Environment of the European Union, Europeans are currently producing an average of 6 tonnes of

waste per person per year (including household and equivalent industrial wastes and wastewaters). This accounts for a total production of 2.5 billion tonnes of waste just in Europe in 2010. From this total amount, only a 36% was recycled while the rest was burned or disposed (European Commission-Environment, 2016b). The waste generated by the industrial sector was 1.3 billion tonnes out of 2.5 billion tonnes in Europe in 2010, which 28% corresponds to the manufacturing industries, including food, beverages, paper, plastics, and many other products (European Statistics, 2010).

Resource recovery is a key point for the Europe 2020 strategy about sustainable growth. What is more, waste management is the second largest contributor to employment advance in the environmental economy (European Statistics, 2016). The European Union Action Plan for the Circular Economy covers the whole product cycle from production to waste management (Figure 1.1). Recycling and reuse are proposed as key actions that will contribute to close the loop. This means that turning wastes and wastewaters into feedstocks and resources will be a key point for achieving the circular economy goal (European Commission-Environment, 2016c).

1.1.2 Resource Recovery from Waste and Wastewater

Following the concept of the circular economy, the European Union is promoting the use of wastewater as a resource for the obtainment of value added products. Researchers have done a huge effort throughout the past 50 years related to the topic of resource recovery, especially in the field of wastewater. The main value added products that have been reported by literature and obtained from wastewater are biogas, organic acids, alcohols, and biopolymers. Very different kinds of waste streams can be used to obtain these recovery products, for example, wastewater sludge, several types of industrial wastes, agro-food wastes, etc.

In the present thesis, resource recovery is focussed on the production of biogas, volatile fatty acids and polyhydroxyalkanoates (PHAs). The processes studied for their production are going to be further developed along the Introduction section.

1.2 BIOGAS

Anaerobic biological processes have been successfully used for several decades and they are one of the most used and successful wastewater treatments to date. P. L. McCarty published in 1964 a series of review articles about Anaerobic Waste

Treatment (McCarty, 1964a; b; c; d). This compilation is still very relevant nowadays for the better comprehension and characteristics of the process even though the technologies he proposed have evolved significantly. Indeed, anaerobic waste treatment has the distinction of being one of the first biological processes used for the valorisation of residues with the obtainment of biogas.

On the one hand, data taken from the ISI web, the number of scientific articles published in referenced journals increased by 10 times from 1991 to 2007, demonstrating the importance of this kind of investigation (Angelidaki et al., 2009). On the other hand, the number of biogas plants in Europe increased from 6,227 in 2009 to 17,376 in 2015. The European biogas plants in 2015 equate to a production of electricity of 60.6 TWh, which corresponds to the consumption of nearly 14 million European households in one year (European Biogas Association, 2016). Germany leads the ranking with 10,846 biogas plants in 2015 (European Biogas Association, 2016)while Spain just treats about 10% of its organic waste by anaerobic digestion (AD) (Abbasi et al., 2012). However, most of the digesters use specifically grown crops instead of wastes, like manure or slurry (Cave, 2013).

1.2.1 Anaerobic Digestion Fundamentals

The AD is predominantly used for the treatment of high-strength organic residues (Tchobanoglous et al., 2003). At the beginning, the AD was applied to complex feedstocks with a containing a variety of nutrients and alkalinity sources (Speece, 1983). Yet in the 90s, the AD process was successfully implemented worldwide (Lettinga, 1995). The success and fast growing of this technology is mainly attributed to its relative simplicity and low cost implementation with the benefit of producing a value added product like biogas and small biomass yields (Lettinga, 1995; McCarty, 1964a; Tchobanoglous et al., 2003). It also contributes to the stabilization of the treated sludge by destroying most of the pathogens, which contains. Generally, the AD operates in the mesophilic range (~35 °C) although its performance in the thermophilic (~55 °C) and psychrophilic (~15 °C) ranges has been also studied.

The AD process takes place, in general, in four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1.2). During the hydrolysis, the complex compounds contained in the wastewater, basically lipids, proteins and carbohydrates, are degraded by bacteria into low molecular weight compounds such

as organic acids, aminoacids and monosaccharides, respectively. In the next stage, acidogenic bacteria degrade monosaccharides and amino acids into mixed organic acids, hydrogen and carbon dioxide. In the third stage, acetogenic microorganisms transform organic acids into acetate, hydrogen and carbon dioxide. The last stage is the methanogenesis where methane is produced by means of the consumption of hydrogen by hydrogen-utilising methanogenic group and the consumption of acetate by aceticlastic methanogenic groups (Batstone et al., 2002).

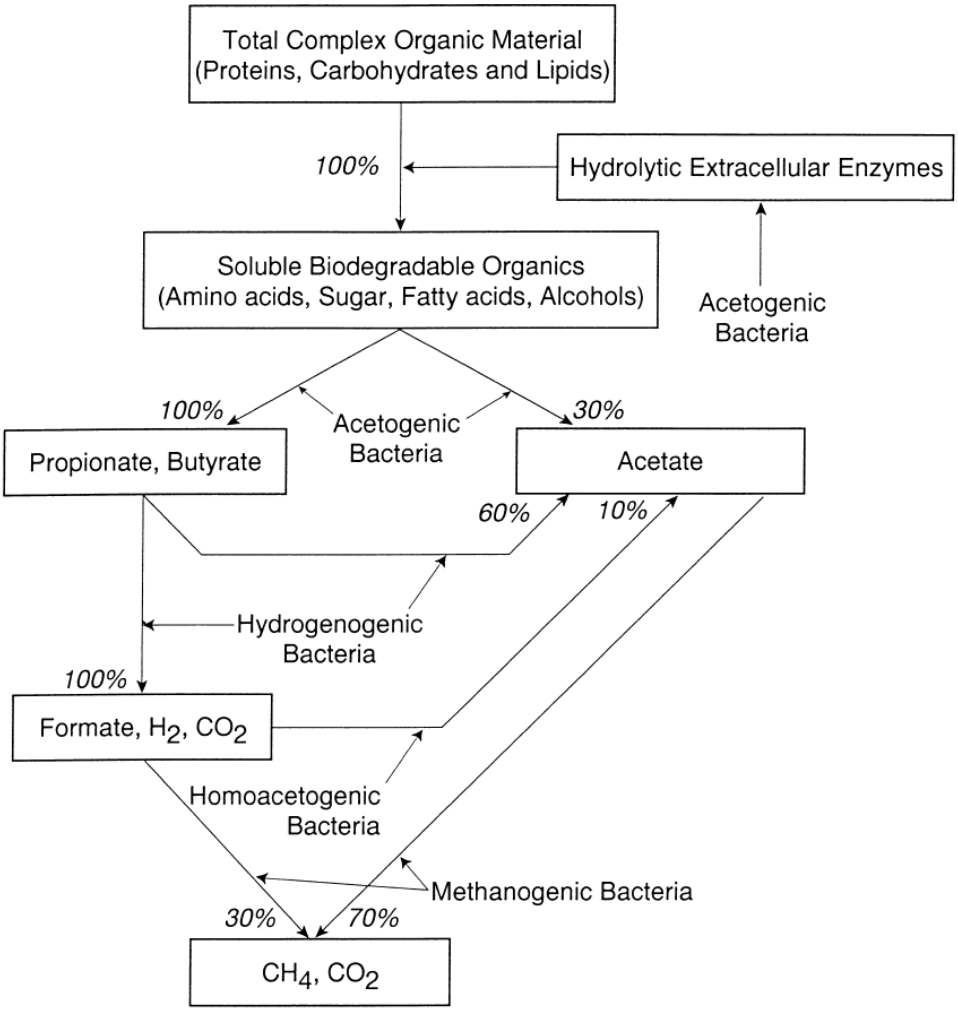


Figure 1.2. Stages of the anaerobic digestion process (Husain, 1998).

1.2.2 Potential Feedstocks

Traditionally, the AD was used for the treatment of sewage sludge and/or animal manure (Weiland, 2010). Nowadays, it is known that several types of feedstocks can be used as substrates for the production of biogas although its composition and the methane yield are strongly dependent on the type of substrate, the type of used technology and operational parameters like the retention time (Braun, 2007). For example, lipids lead to higher biogas yields although they require longer retention times, while carbohydrates and proteins need shorter retention times but, on the other hand, lower biogas yields are achieved (Weiland, 2010).

Types of substrates that have been found suitable to be treated by AD is extensive. Examples of typical substrates for the AD are sewage sludge (Appels et al., 2008; Astals et al., 2013; Zhen et al., 2017), food wastes (Li et al., 2017; Rajagopal et al., 2017; Zamanzadeh et al., 2016), soft-drink bottle industry effluents (Ghosh et al., 1985), very different varieties of crops (González-González et al., 2013; Goulding and Power, 2013), seafood and fish-canneries (Feijoo et al., 1995; Soto et al., 1993), manure (Hadin and Eriksson, 2016; Hansen et al., 1998; Pessuto et al., 2016), etc.

This topic is such extensive that, for further information, it is recommended to look after a specific substrate, the configuration of the system and the applied operational conditions to know the feasibility of the process and the biogas potential. Nevertheless, it is important to remark that nowadays the mono-digestion has lost importance in favour of the co-digestion. Mixing substrates for the enhancement of the methane production during the AD process is being the focus of attention within this field as it overcomes several weaknesses and drawbacks of the traditional AD process while improving its economic feasibility (Mata-Alvarez et al., 2014).

1.2.3 Operational Parameters and Inhibitors

It is well-known that factors like pH, temperature, organic load, alkalinity, and type/nature of the feedstock, among others, affect the performance of the AD process (Appels et al., 2008; McCarty, 1964b).

The microorganisms involved in the AD process have different optimum pH although methanogenic ones are the most sensitive to these changes. For example, the optimum range for methanogens is between 6.5 and 7.2 while acidogenic microorganisms can function between 4.0 and 8.5 (Appels et al., 2008). Alkalinity

and volatile acids concentration play an important role in the pH balance. Indicators of the increase of the concentration of acids are the decrease of pH and the increase of CO_2 in the gas phase. This behaviour is due to the depletion of the bicarbonate, which acts as buffer in the system (Ripley et al., 1986). So as to control this, alkalinity is frequently measured in order to stablish the appropriated measures to recover the optimum reactor performance.

The temperature of the reactor is also important since it affects the metabolism of microorganisms and its augmentation decreases the solubility of gaseous compounds. Even some beneficial reactions would be thermodynamically favoured and the death of pathogens would be improved with the increase of temperature (Rehm et al., 2000).

Both hydraulic retention time (HRT) and solids retention time (SRT) are important for the performance of the AD process. If the SRT value is decreased, the reactions taking place occur in less extent and a larger fraction of the sludge, performing the AD inside the reactor, is removed during the effluent withdrawal (Appels et al., 2008). Appels et al. (2008) stated that SRTs shorter than 5 days led to an unstable AD process while this process becomes stable for SRTs longer than, at least, 8-10 days. Nevertheless, and despite all the recognised advantages of the process, the anaerobic digestion has some limitations. For example, this process is characterized by slow reaction rates and sensitivity to potential inhibitors, like salinity associated to sodium chloride (Appels et al., 2008) or other ions, the presence of high ammonia concentrations, and sulphide.

Ammonia is produced by the degradation of proteins contained in the wastewater. It is present in the liquid phase in the form of ammonium (NH_4^+) or free ammonia (NH_3). The inhibitory form of this compound is the free ammonia since it is freely membrane-permeable (Chen et al., 2008). However, a previous research study indicated that full recovery of the methanogenic process is feasible within 24 h after the non-inhibitory ammonia levels are restored, with no remarkable difference between the responses of an acclimated and a non-acclimated biomass (de Baere et al., 1984). De Baere et al. (1984) also recommended free ammonia concentrations lower than 80-100 mg/L. Nonetheless, a total ammonia concentration of 10 g/L has been reported as lethal, regardless acclimation (Liu and Sung, 2002). In this inhibitory effect, the pH and temperature of operation play an important role (Anthonisen et al., 1976).

Common inhibitors are ions like Na^+, K^+, Mg^{2+} and Ca^{2+}, among others. It has been observed that the toxicity is usually determined by the cation, although they must be related with their corresponding anions of the coming salt (Chen et al., 2008). Specific Na^+ concentration values are difficult to establish as they are strongly dependent on acclimation and/or presence of other ions (de Baere et al., 1984; Feijoo et al., 1995; Omil et al., 1995; Soto et al., 1993). Chen et al. (2008) summarised in a review article values of the half maximal inhibitory concentration (IC_{50}) corresponding to a value ranging from 5.6 to 53 g/L. This wide range of values might be attributed to the adaptation and antagonistic/synergistic effects, configuration of the reactor, type of substrate, etc. Literature, where the effect of sodium chloride on the AD is researched, reveals noteworthy variations about the toxicity levels and the subsequent inhibition of the process as antagonism/synergism and acclimation influence (Feijoo et al., 1995; Soto et al., 1993). It is important to remark that methanogenic microorganisms are usually considered the most sensitive population to toxicity in the anaerobic consortium, although there are exceptions (Speece, 1983).

Sulphide is another important inhibitor of the AD process. This compound is produced by the reduction of sulphate present in the wastewater in anaerobic environments by the sulphate reducing bacteria (SRB). In principle, sulphide is known to be toxic to some microorganisms (Chen et al., 2008). Nonetheless, there are two other important effects of this compound: (1) SRB compete for the organic matter with the anaerobic microorganisms involved in the AD and in this way biogas production decreases; and (2) the presence of sulphide in the gas phase affects the further uses of the biogas (O'Flaherty and Colleran, 1999; Palmeiro-Sánchez et al., 2013). Again, the reported toxicity values for sulphide are variable, ranging from 150 to 1100 mg/L when referred to dissolved sulphide and from 50 to 250 mg/L when referred to free sulphide (Omil et al., 1995).

Other factors that can inhibit the AD process are catalyst or solvents coming with the influent, pharmaceutical compounds, heavy metals, some organic compounds (benzenes, phenols,…), etc (Chen et al., 2008; Speece, 1983).

1.2.4 Reactors applied for Anaerobic Digestion

The anaerobic reactors are classified according to various categories. For example, Tchobanoglous et al. (2003) classified them as: (1) anaerobic suspended

growth; (2) up-flow and down-flow anaerobic attached growth; (3) fluidized-bed attached growth; (4) upflow anaerobic sludge blanket (UASB); (5) anaerobic lagoons; and (6) membrane separation anaerobic processes. Since this description is one of the most completes one, it is going to be followed in the present document.

Anaerobic suspended growth processes were the first ones in being used. To this category belong the classical continuous stirred tank reactor (CSTR), also called complete mix suspended growth anaerobic digester; the anaerobic contact process; and the anaerobic sequencing batch reactor (ASBR). The complete mix process is characterised by having the same SRT and HRT while the anaerobic contact process is characterised by including a clarifier with the subsequent recycling of biomass. This means that the HRT and SRT values are no longer equal as the SRT is significantly higher than the HRT, so the reactor volume can be reduced. Finally, the ASBR can be considered as suspended growth contact process but with the reaction and separation phases occurring in the same vessel. Typical values for the organic loading rates (OLRs) at 30 °C for a CSTR are between 1.0 - 5.0 kg COD/(m^3 · d), while these values are in the range of 1.2 – 2.4 kg COD/(m^3 · d) for an ASBR (Tchobanoglous et al., 2003).

Attached growth reactors just differ in the type of packing material and the degree of bed expansion, which ranges from 20 to 100 per cent. In the up-flow packed bed reactor, the packing is fixed and the wastewater flows through the interstitial space. Removal efficiencies up to 90% are achieved at OLRs of 1.0 to 6.0 kg COD/(m^3 · d) for wastewaters like food canning ones (Young, 1991). Other types of attached growth reactors are the anaerobic expanded-bed (AEBR) and the fluidized bed (FBR) ones. The packing material for the AEBR process has usually been silica sand of 0.2 – 0.5 mm of diameter since the small packing make available higher surface area per unit of volume, supporting a greater amount of biomass (Tchobanoglous et al., 2003). The application of the AEBR has usually been the treatment of domestic wastewater, even at low temperatures, achieving removal efficiencies of about 80-89 % at 15-20 °C and even 71 % at 10 °C (Alderman et al., 1998). For the FBR process, sand of about 0.3 mm is used at high up-flow velocities, achieving 100 % of bed expansion. Nevertheless, other packing materials have been used like activated carbon, diatomaceous earth, etc (Tchobanoglous et al., 2003). Removal efficiencies up to 99 % have been achieved with FBRs treating starch and whey at 35 °C at a OLR of 8.2 kg COD/(m^3 · d). Even removal efficiencies of 70 % were achieved when treating citric acid at a OLR of 42 kg COD/(m^3 · d) (Denac and

Dunn, 1988). The development of the UASB reactor by Lettinga in the '70s was one of the most notable developments in the anaerobic treatment technology. It has been developed to treat high organic loading rates. Removal efficiencies of 90 – 95 % were achieved when treating wastewaters at 30 – 35 °C at OLRs of 12 – 20 kg COD/($m^3 \cdot d$) at HRTs as low as 4 – 8 hours (Tchobanoglous et al., 2003). Large concentrations of biomass in the form of granular sludge can be kept inside the reactor, which are higher than those treated in suspended biomass technologies. The UASB reactors are also characterised by a gas-solid separator located in the upper part of the reactor.

The anaerobic digestion can also take place in ponds and lagoons to treat wastewaters but which require huge surface for implantation, although the costs of construction and maintenance are low. Typical OLRs are in the range of 1.0 – 2.0 kg COD/($m^3 \cdot d$) with HRTs of 20 – 50 days and SRTs up to 100 days (Tchobanoglous et al., 2003). Finally, the use of membrane reactors is one of the newest alternatives under development among the anaerobic treatment technologies. These systems allow the maintaining of high biomass concentration while giving a significantly good-quality effluent. These versatile systems are able to treat OLRs in the range of 2.0 – 22.0 kg COD/($m^3 \cdot d$) with HRTs of 0.5 -o 15 days while high SRTs are kept (30 – 160 days) (Tchobanoglous et al., 2003).

During AD, the produced biogas is typically used for thermal or chemical energy applications. It can be directly used for the production of heat or electricity or even in Sweden is employed as a fuel for vehicles (Kadam and Panwar, 2017).

1.3 VOLATILE FATTY ACIDS (VFAS)

Following the aim of resource recovery, the application of anaerobic processes for the treatment of wastewater can provide very useful value-added products for the chemical industry (Lin et al., 2013), like volatile fatty acids (VFAs). VFAs are produced during the acidogenesis, as indicated in Figure 1.2 (Husain, 1998), corresponding to the second stage of the AD, in which organic acids and alcohol are produced (Cohen et al., 1979). There are several developed methods for the production of acids although they differ among them as the concentration, yield and composition of the produced VFAs is affected by operational parameters (Lee et al., 2014) but also by the substrate (Silva et al., 2013). These VFAs are valuable products than can be further used for the generation of bioenergy (electricity through

microbial fuel cells, production of hydrogen…) or even for biopolymer production (Lee et al., 2014).

1.3.1 Potential Feedstocks

Wastes can be grouped into several categories like industrial, agricultural, sanitary and solid urban residues (Lin et al., 2013). Several solid and liquid wastes have been already studied as potential feedstocks for VFA production like primary sludge, solid waste, paper mill effluent, cheese whey and many others (Lee et al., 2014; Silva et al., 2013). Several types of wastes together with their initial content in COD and the corresponding VFA production can be seen in Table 1.1.

Table 1.1. Summary of residues used for VFA production (adapted from Lee at al. (2014); Silva et al. (2013)).

Type of Waste	Initial Organic Content (g COD/L)	VFA Production
Primary Sludge	0.34	0.03 g VFA/(g VSS d)
	20.6	0.06 g COD_{VFA}/(g VSS d)
Olive Oil Mill Effluent	37.0	10.7 g COD_{VFA} /L
	70.4	15.6 g COD_{VFA} /L
	8.13	0.93 g COD_{VFA} /L
Wood Mill Effluent	11.1	0.37-0.42 g COD_{VFA} /g $COD_{influent}$
Paper Mill Effluent	8.8	0.74 g COD_{VFA} /g COD
	26.3	0.60 g COD_{VFA} /g COD
Dairy Wastewater	4.0	1.03 g VFA/L
	12.0	2.07 g VFA/L
Waste Activated Sludge	14.9	0.19-0.34 g COD_{VFA} /L
Pharmaceutical Wastewater	40-60	0.44 g COD_{VFA} /g $COD_{influent}$
Cheese Whey	8.05	3.37 g COD_{VFA} /L
Sugarcane Molasses	8.10	3.11 g COD_{VFA} /L
Wasted Glycerol	8.13	1.46 g COD_{VFA} /L
Winery Effluent	8.01	1.14 g COD_{VFA} /L
Landfill Leachate	8.03	0.24 g COD_{VFA} /L

1.3.2 Operational Parameters and Inhibitors

Operational parameters like retention time, pH, type of substrate, C/N ratio, and temperature, among others, have pronounced influence on VFA production. Changes in these parameters affect the composition of the produced VFAs and on the concentration and the stoichiometric and kinetic parameters corresponding to the biological reactions.

Both HRT and SRT are of main importance in the acidogenic fermentation to produce VFAs. The HRT defines the design of the reactors and the operational costs while the SRT rules the selection of the microbial community involved in the process (Lee et al., 2014). These retention times clearly influence both VFA composition and production, but this influence is dependent on the biomass or wastewater characterisation, as there are substrates more easily fermentable than others. For example, cheese whey is mainly composed by lactose, which is a readily fermentable compound, while pulp and paper mill wastewater degradation depends more on the origin of the feedstock and the type of process taking place, since raw materials may contain relevant amounts of lignin (Bengtsson et al., 2008). It has been observed that high retention times lead to high VFA concentrations, although methanogens might grow under the same operational conditions. For example, at a pH of 10, a retention times of 5 days hardly led to 0.3 g VFA/g VSS while a retention time of 15 days led to values close to 0.6 g VFA/g VSS (Jankowska et al., 2015).

In the case of the pH, it is known that values lower than 3 or higher than 12 are inhibitory for the acidogens (Khan et al., 2016; Liu et al., 2012). The optimal value might range between 5 and 11, depending on the type of waste (Lee et al., 2014). Nevertheless, the pH not only affects the VFA yield but also the acetate, propionate, butyrate and valerate distribution in the liquid phase (Bengtsson et al., 2008).

The effect of temperature over VFA production has been studied in the psychrophilic, mesophilic and thermophilic ranges, like biogas production. Temperature is also crucial and it seems that its increase promotes VFA production along with VFA concentration and yield although this influence is minor in comparison with the effect of the pH (Lee et al., 2014).

The C/N ratio is a critical parameter since both carbon and nitrogen are elemental nutrients for the cellular maintenance and growth. The C/N ratio affects the yield significantly and the highest yield (0.36 g VFA/g VS) was achieved at C/N

ratios between 13 – 25 g C/g N (Rughoonundun et al., 2012). These authors also found that the C/N ratio affected the distribution of the VFAs. C/N ratio values as high as 87 g C/g N inhibit the production of acids (Silvestre et al., 2015).

The effect of inhibitors over the acidification activity has been studied as part of the anaerobic treatment, although these studies are usually more focussed on the methanogens inhibition, since they are more sensitive to fluctuations on the operational parameters like ammonia concentration, overloading, C/N ratio, etc. Regarding the effect of NaCl over the VFA production, it has been found that high concentrations of sodium chloride affect the acidification negatively when treating kitchen waste (Lee et al., 2015). However, the NaCl addition when fermenting waste activated sludge enhanced the VFA production, promoting the n-butyric and decreasing the propionic concentration (Su et al., 2016). Su et al. (2016) achieved concentrations from 3 to 30 mol NaCl/L and they justify this beneficial behaviour by explaining that the presence of NaCl inhibited the methanogenic activity. Nonetheless, it seems that this field must continue being explored.

1.3.3 VFAs Production and Applications

The processes for VFA production are based either on attached or suspended growth (Tchobanoglous et al., 2003) which lead to different reactor configurations. The most used technologies (Figure 1.3) (Khan et al., 2016; Lee et al., 2014) are: (a) packed bed reactors which have the biomass attached to a packing material; (b) fluidized bed reactors with the biomass attached to small particles (i.e., sand) that are in suspension and in motion in the fluid; (c) UASBs are suspended growth reactors with granular biomass that can easily settle and equipped with a separator for the gas-liquid-solid phases; (d) CSTRs characterised by the complete mixing between microorganisms and substrate.

The effluent rich in VFAs can be used for some applications or they can be recovered. The choice depends on their further use. VFA-rich streams can be utilized as substrate to produce bioplastics, the conversion to alcohol fuels or biodiesel, to generate bioenergy or to carry out biological nutrient removal, among other applications (Silva et al., 2013). Pure VFAs are widely used in pharmaceutical, textile and plastic industries (den Boer et al., 2016).

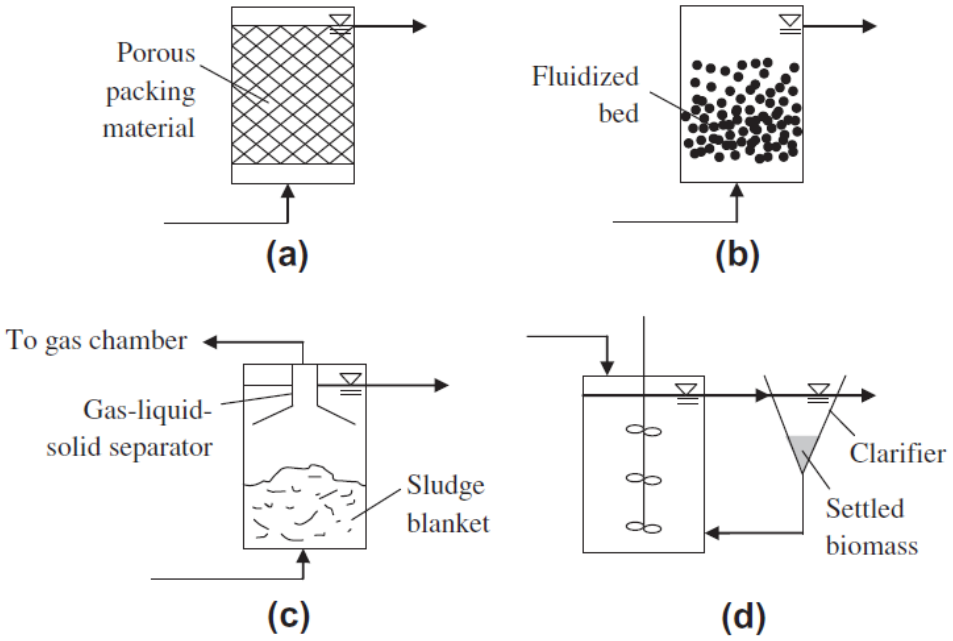


Figure 1.3. Schemes of fermenters used for VFA production: (a) Packed bed reactor; (b) FBR; (c) UASB; and (d) CSTR (Lee et al., 2014).

1.4 POLYHYDROXYALKANOATES (PHAS)

Plastics produced from crude started to be present in our daily lives about one hundred years ago, so they can be considered as modern materials compared with glass, paper, etc.

At the beginning of the 20th century, many materials (such as solvents, dyes, fibres, etc.) were made from natural sources. But in the last fifty years the pattern has changed and, in the middle-end of the 60s, the petrochemical industries displaced completely most of the bio-based productions and petroleum derivatives began to rule the world in every single way. Few years later, during the 70s, an energy crisis started and many industrial countries had to deal with shortages of petroleum because of the high increase in the petroleum prices (Braunegg et al., 1998; Ragauskas et al., 2006). The two mayor peaks in production during the 20th century occurred in 1973 with the Yom Kippur war and 1980 with the Iran-Iraq war. Then the Global Crisis which started in 2008 was the cause of the highest prices ever registered (Figure 1.4). This energy crisis, which started on the 70s, was useful to

drive the study of new processes for the synthesis of plastics from bio-resources of fuels and other petroleum-based materials (Ragauskas et al., 2006).

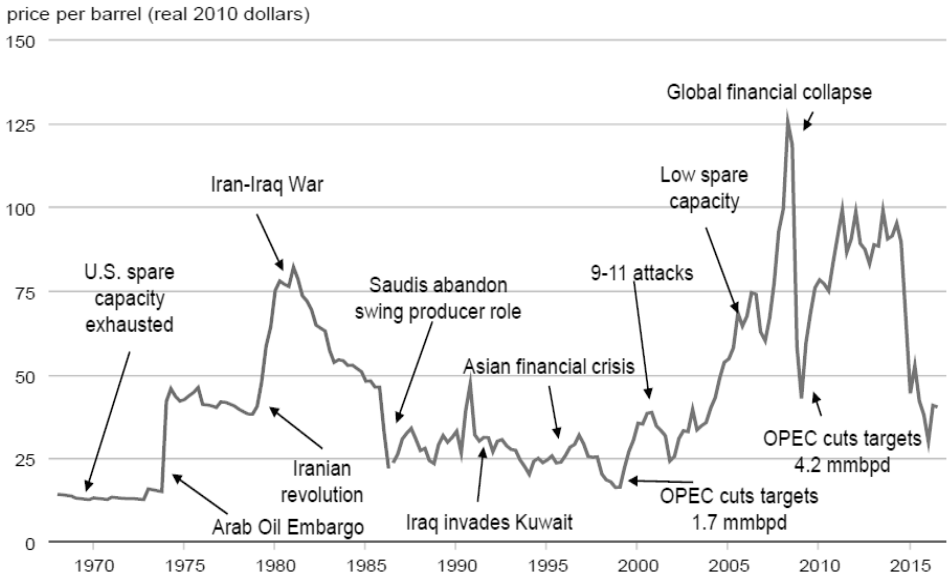


Figure 1.4. Evolution of the price of a barrel of oil in US dollars (US Energy Information Administration, 2016).

Nowadays plastics have many uses and are essential for the modern life. They have unimaginable uses in building, construction, packaging, medical applications, electronic components, sport materials and any other conceivable utility. The consumption of plastics in Western Europe and North America is estimated about 100 kg per person per year and nearly 300 million tons of plastics per year are produced worldwide (Figure 1.5). The upward trend is still maintained despite the Global Crisis of 2008.

Plastics are so extensively used because they offer a lot of advantages over other typical materials such as paper or wood. Among many other useful properties, they are light, cheap, durable, resistant and versatile.

The waste-treatment of plastics depends on the country but usually the most common options are reutilization, recycling, incineration and landfill. Unfortunately, at this moment there is no uniform waste management practice for the plastics in the European Union (Figure 1.6) and a lot of countries are very far from sustainable goals.

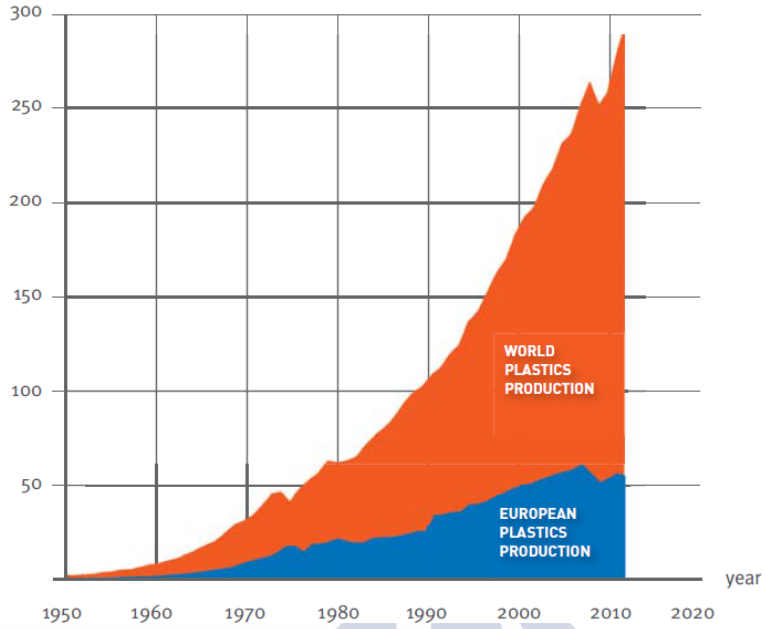


Figure 1.5. Plastics production from 1950 to 2012 (PlasticsEurope - Association of Plastics Manufacturers, 2013).

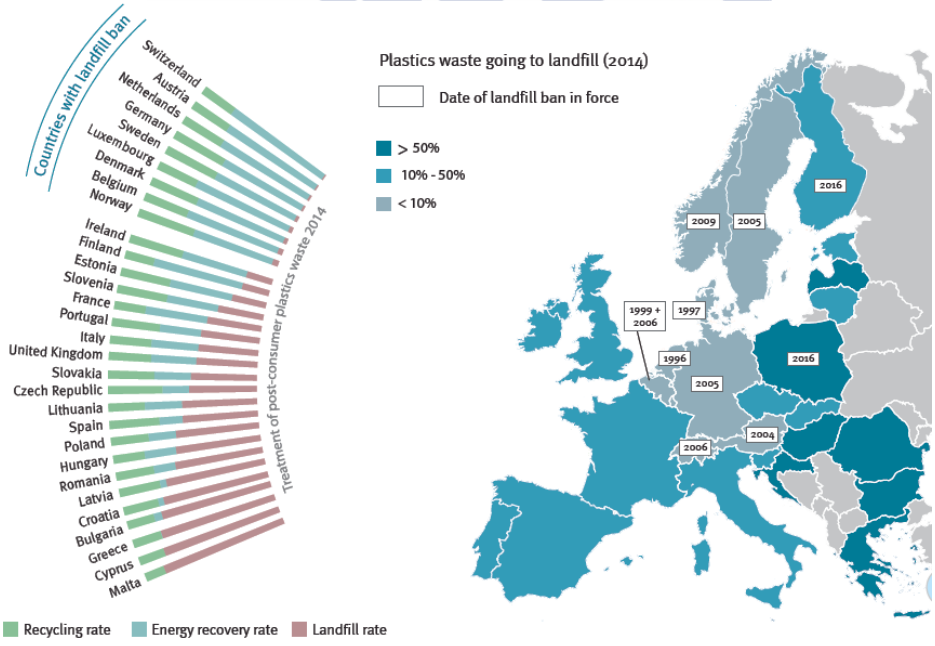


Figure 1.6. Plastics recovery ratio in the European Union by country in 2014 (PlasticsEurope - Association of Plastics Manufacturers, 2016).

In conclusion, plastics come from crude oil which is not sustainable because of its shortage. Another reason is that the manufacturing process is not environmentally friendly because of the greenhouse gas emissions associated. Finally, plastics can persist in the environment for hundreds of years, having disastrous consequences for the ecosystems. For example, in the Central North of the Pacific Ocean, i.e., the "Great Pacific Garbage Patch" exists with an estimated size between twice the size of Texas to twice the size of the United States. It probably contains around 100 million tons of wastes**.** To diminish this effect the progressive substitution of conventional plastics by bioplastics seems to be a feasible alternative.

Nowadays, the expression "bioplastic" is quite familiar to all the individuals in our society. Despite the fact that this term is widely accepted, there is not a general agreed definition yet. A wide classification admits that bioplastics are classified as bio-based and/or biodegradable products (Ashter, 2016) but not necessary the two situations simultaneously. This means that the word "bioplastic" can refer to groups of very different types of plastic materials (Figure 1.7). It includes plastics derived from renewable materials and biobased feedstocks but non- or hardly-biodegradable (i.e., polyamide). It also covers biodegradable plastics made from non-renewable materials (i.e., Ecoflex® of BASF). And, finally, bioplastics can also be referred to biobased plastics that are biodegradable (i.e., starch and soybean plastics, but this type of plastics need some additives to improve their properties). In this last group, bacterial plastics (i.e., PHAs) are included.

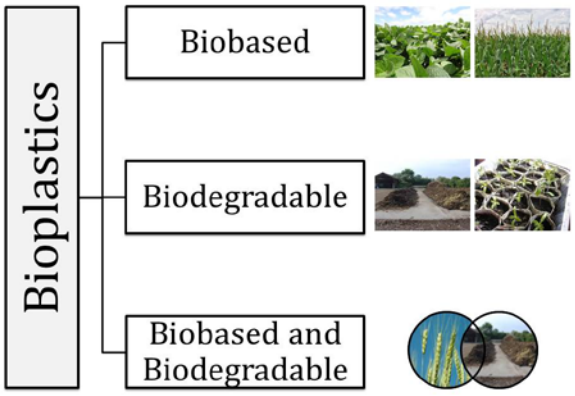


Figure 1.7. Different types of bioplastics depending on their properties.

With respect to the group of biobased and biodegradable bioplastics, microorganisms can produce the needed monomers (building blocks) to form these bio-based polymers. The most common of these building blocks are shown in Figure 1.8 together with their corresponding polymer units.

The importance of the PHAs relies on the fact that this is the only biopolymer completely synthesized by microorganisms and, furthermore, it does not normally appear as a single monomer (Chen, 2010b). In strict terms, PHAs can be considered as the only fully bio-based and biodegradable plastic polymers because they are directly produced by microorganism, fully biodegradable and can be made from 100% renewable resources (Dias et al., 2006).

There are more bacterial plastics and all of them can be used for similar purposes as some crude-oil based plastics, depending on their properties. For example, bio-polyethylene has the same characteristics as conventional polyethylene (Chen, 2010b); and polyhydroxybutyrate (PHB) is usually compared to polypropylene (Kaplan, 2000)although PHB is more rigid and brittle. Polyethylene is the less biodegradable of these three bacterial plastics but Polyhydroxyalkanoates and Polylactic Acid are well known for their biodegradability (Chen, 2010b). Both Polyhydroxyalkanoates and Polylactic Acid lead to thermoplastic polymers.

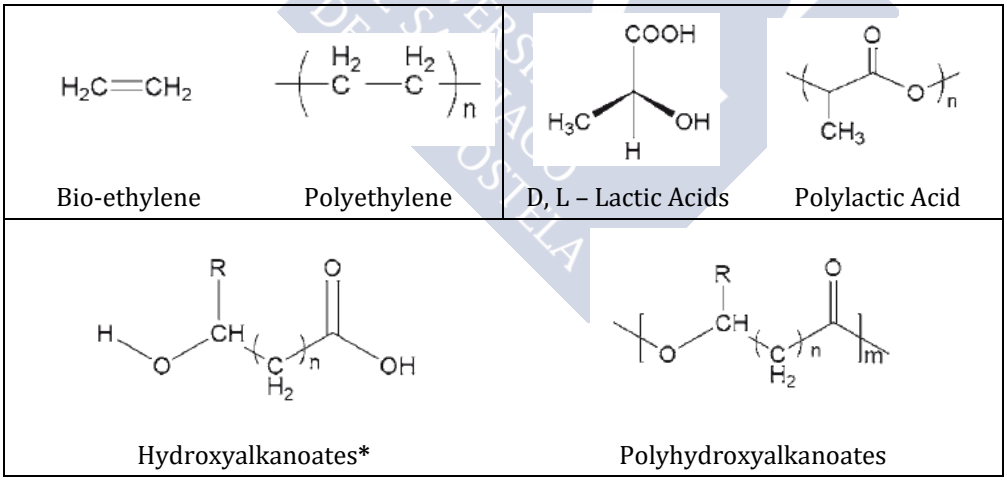


Figure 1.8. Most common monomers in bacterial plastics and their corresponding polymer units.

*PHA do not usually appear as a monomer (Chen, 2010b)

PHAs are granules that can be easily seen with a microscope just with simple staining techniques inside the bacterial cells. The very first observation of this PHA aggregates was done by Beijenrinck in 1888 (Rathna et al., 2016). Nevertheless, the discovery of PHB production, as a kind of PHA, was carried out by Lemoigne in 1927 using a pure culture of the specie *Bacillus megaterium* (Volova, 2004). Since then, hundreds of different types of pure culture microorganisms have been reported as natural PHA producers (Steinbüchel and Valentin, 1995) including some genetically-modified ones (Steinbüchel and Füchtenbusch, 1998; Sudesh et al., 2000). As early as 1982, the first studies indicated that the properties of poly-3-hydroxybutyrate (P3HB) were similar to those from the polypropylene (King, 1982). Also in this year another research study indicated the biodegradability and biocompatibility of the P3HB (E, 1982).

The challenge now for researchers is to improve the process to reduce these production costs. One way is based on the use of wastes as feedstocks. Another is the use of mixed microbial communities instead of pure cultures but achieving the same production yields (Jiang et al., 2012; Reis et al., 2003). Also the extraction process should be improved because it has a high impact in the costs (Madkour et al., 2013) and the environment.

The synthesis of PHA under non-sterile conditions, in mixed culture systems, was observed for the first time associated to the biological phosphorous removal in wastewater treatment plants where alternating aerobic and anaerobic cycles were used (Wallen and Rohwedder, 1974). Majone et al. (1996) also found that accumulation of PHA occurred when periods of excess and limitation (feast/famine) of carbon source where present in a system (Majone et al., 1996). Many bacteria in extreme environments are also able to store PHA. Bacterial populations that can use these storage compounds as reserve and support substance, i.e., in starvation conditions have competitive advantage over those without this capability (Castro-Sowinski et al., 2010) facilitating their enrichment. When appropriated selection conditions are imposed to a mixed culture, the accumulating bacterial populations will overgrow those unable to accumulate PHAs.

1.4.1 Potential Feedstocks

The most common substrates for pure culture processes are glucose or other sugars and also organic acids, which elevates the production costs up to 50%

(Braunegg et al., 2004). Nevertheless, zero-impact during PHA production will be possible if residues are used as feedstocks and microbial mixed cultures are used. In this way the precepts of the circular economy will be closer (European Commission-Environment, 2016d). In this way, valuable products are obtained while a residue is treated. It is known that using a waste as feedstock allows a reduction of the production costs be up to 50% (Choi and Lee, 1997). Different types of residues are good candidates for being used for PHA production (Figure 1.9.) either in pure and mixed cultures: lipidic wastes, biofuel production wastes, surplus whey from the dairy industry, lignocellulosic wastes, residues from the sugar industry, etc. (Nikodinovic-Runic et al., 2013).

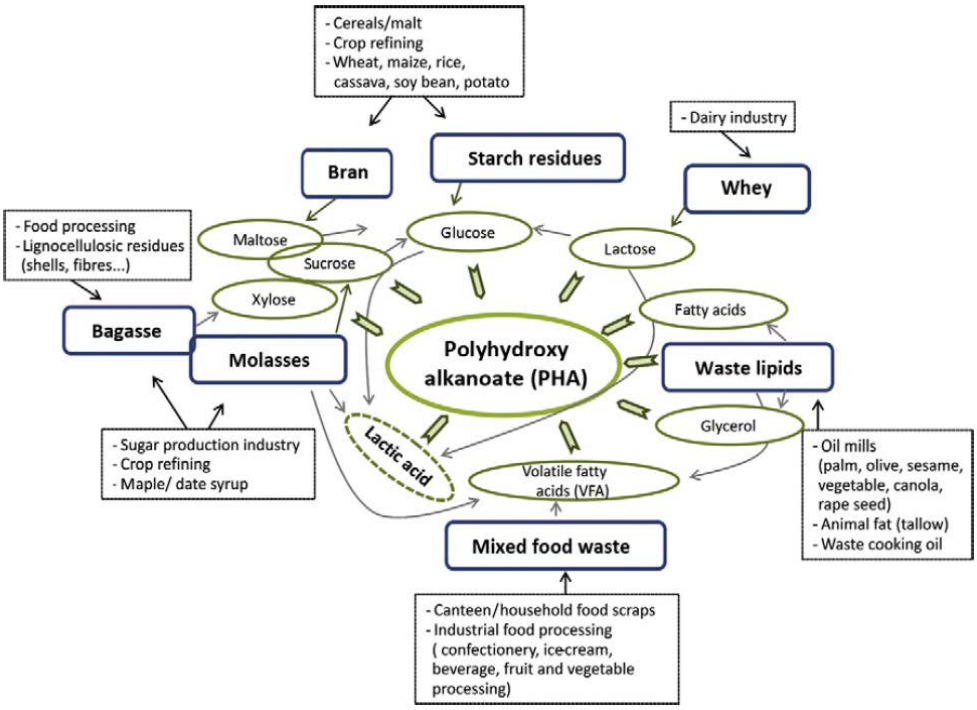


Figure 1.9. Residues that can be used as feedstocks for PHA production (Nikodinovic-Runic et al., 2013). Most of them required the obtaining of VFAs in a first stage.

When using complex carbon sources as substrates, a stage previous to the first one is needed. This preliminary reactor is a fermentation of the wastewater so as to produce volatile fatty acids (Albuquerque et al., 2011; Duque et al., 2014; Jia et al.,

2014). Lipidic wastes include cooking oils and residues from the palm oil (Din et al., 2012) or olive oil industries (Dionisi et al., 2005; Waller et al., 2012) among others. Din et al. (2012) achieved PHA yields of 0.80 Cmol PHA/Cmol VFA for fermented palm-oil effluents while Dionisi et al. (2005) achieved values of 1 g PHA/g VFA on COD basis in the first hours of the accumulation experiments. This fermentation step is not required for substrates like glycerol (Moralejo-Gárate et al., 2011).

Residues from the sugar industry have also been studied for a long time for PHA production (Albuquerque et al., 2007; Albuquerque et al., 2010; Bengtsson et al., 2010; Gouda et al., 2001). Albuquerque et al. (2010) achieved a yield of 0.65 g PHA/g substrate when using fermented molasses as influent for biopolymer production, which means a 75 wt% of PHA accumulated inside the cells.

Biofuel production wastes include, for example, PHA production from glycerol waste-streams from the production of biodiesel (Lemos et al., 2014; Moita et al., 2014; Moralejo-Gárate et al., 2011). Glycerol does not need a previous fermentation, unlike the other substrates presented previously. Moralejo et al. (2011) obtained an average yield of 0.40 g PHB/g glycerol which it is equivalent to an accumulation of 67 wt% of PHB. Whereas Moita et al. (2014) achieved 0.51 Cmol PHB/Cmol glycerol in the case of using synthetic glycerol and 0.46 Cmol PHB/Cmol glycerol in the case of using crude glycerol as substrate which is equivalent to accumulation values of 47 wt% of PHA and 53 wt% of PHA, respectively.

1.4.2 PHA production in mixed microbial cultures

When PHAs are produced with pure culture sterile conditions are mandatory (Lee, 1996; Serafim et al., 2008) which increase the total production costs up to 11% (Venkateswar Reddy and Venkata Mohan, 2012). The use of microbial mixed cultures would be of interest in some cases to lower the costs since aseptic conditions are not needed, making possible the use of inexpensive substrates (like waste streams) can be used.

The initial idea of producing PHAs with mixed communities appeared by observing activated sludge processes. This type of process is highly dynamic where microorganisms access the substrate just during short periods of time. Microorganisms able to store reserve substances have a competitive advantage in comparison with organisms that are not able to accumulate them (Castro-Sowinski et al., 2010). PHAs are stored inside the cells as granular inclusions (Serafim et al.,

2008) under stress conditions caused by nutrient limitation (Anderson and Dawes, 1990; Dionisi et al., 2004a; Dionisi et al., 2004b; Majone et al., 2006; Serafim et al., 2004).

The selection of mixed microbial cultures used to produce PHAs is performed in general in an enrichment reactor. This enrichment is achieved by means of the application of a selective pressure, which takes advantage of the ecologic role of PHAs as reserve material (van Loosdrecht et al., 1997). Two different strategies can be followed (Johnson et al., 2010b; Reis et al., 2003):

- Aerobic-anaerobic sequence

  This strategy alternates periods of presence and absence of an electron acceptor (dissolved oxygen) with the supply of substrate during the absence of the electron acceptor. This strategy is for microorganisms able to store glycogen and/or polyphosphate (Johnson et al., 2010b). In aerobic conditions, the accumulated PHAs are used for growth and maintenance while glycogen or polyphosphate storage reserves are replenished again. Under anaerobic conditions, the substrate is used for PHA production while glycogen or polyphosphate is consumed. These microorganisms store about 20 wt% PHA, although maximisation was endeavoured (30-57 wt% PHA) but no constant values were obtained for the PHA storage capacity (Serafim et al., 2008).

- Feast-Famine regime

  The feast-famine regime or aerobic dynamic feeding (ADF) is referred to the alternating presence and absence of carbon source in the liquid phase under excess of nitrogen source. The accumulation capacity under ADF is enhanced by the use of sequencing reactors (van Loosdrecht et al., 1997). This strategy promotes the community enrichment in microorganisms with high accumulating capacity (Dionisi et al., 2005; Jiang et al., 2011; Johnson et al., 2010a; Moralejo-Gárate et al., 2011). These processes which follow the strategy of limiting the carbon source operate in two stages: the first one for the selection of the mixed microbial community applying the selective pressure of the ADF and, the second one, for the maximisation of biopolymer accumulation inside the cells by limitation of the nitrogen source. The PHA accumulation in dry weight during the maximisation stage

is very high, reaching values of nearly 90 wt% PHA (Johnson et al., 2009) which are close to the values achieved by pure cultures (Moralejo-Gárate et al., 2011; Serafim et al., 2004).

1.4.3 Operational Parameters and Inhibitors

When mixed microbial cultures are used, the PHA composition and physico-chemical properties depends on the operational conditions. Although, these properties have been studied in previous research works, there is little information about the influence of the operational parameters.

Some of the most important influencing factors are: carbon source concentration and composition, alternation of different feedings, temperature, pH, media composition, extraction method or even by use of dynamic conditions like the ADF (Albuquerque et al., 2011; Arcos-Hernández et al., 2013; Hilliou et al., 2016; Palmeiro-Sánchez et al., 2016b; Serafim et al., 2004; Villano et al., 2010).

Nevertheless, in many cases the variability of the PHA properties produced by mixed microbial communities is mainly attributed to the substrate composition. Different copolymer compositions can be achieved even when using the same substrate composition depending on the properties of the enriched mixed microbial community (Albuquerque et al., 2013; Carvalho et al., 2014; Lemos et al., 2006). According to Albuquerque et al. (2013), *Azoarcus* consumes preferably acetate and propionate, *Thauera* has preference for propionate and butyrate, while *Paracoccus* is able to consume any volatile fatty acids. Carvalho et al. (2014) observed that the abundance of the different bacterial genera lead to diverse biopolymer compositions and also to different amounts of biopolymer inside the cells. These authors proved that greater amounts of *Azoarcus* lead to superior PHA production yields and lower microorganism growth in comparison with communities where the predominant genera were *Thauera* and/or *Paracoccus* (Carvalho et al., 2014).

The effect of possible inhibitors has been scarcely studied in PHA production. In the present thesis, the influence of the presence of sodium chloride as a possible inhibitor is of interest due to its high concentration in the treated wastewaters (Mendez et al., 1995). It is known that the presence of sodium chloride affects the activity of many different microbial communities in biological wastewater treatment systems (Uygur and Kargı, 2004; Wang et al., 2005). To the knowledge of the authors,

the influence of NaCl in non-adapted communities has been barely studied in pure cultures (Mozumder et al., 2015; Passanha et al., 2014) while just one work has been published in mixed microbial communities (Palmeiro-Sánchez et al., 2016a). However, regarding the effect of NaCl over the pure culture's activity, contradictory results have been reported. On the one hand, a progressive reduction of the accumulated PHB by *Paracoccus denitrificans* and *Cupriavidus necator* using glycerol as substrate was observed at concentrations above 5 g NaCl/L, while an inhibition of 80% occurred at 20 g NaCl/L (Mothes et al., 2007). On the other hand, Passanha et al. (2014) found that a concentration of 9 g NaCl/L was optimal for the accumulation of PHAs with acetic acid as substrate using *Cupriavidus necator*. These authors found that the inhibition occurred at 15 g NaCl/L.

Another important point regarding the effect of NaCl concentrations over the PHA-accumulating activity is to investigate the influence of constant concentrations or transient concentrations of NaCl in the activity of the accumulating biomass, which has not been studied before, and it will also be discussed in the present thesis.

1.4.4 PHA Properties

PHAs can be classified in two main groups: (1) short-chain PHAs, with monomer units of 3-5 carbon atoms, and (2) medium-chain PHAs, with monomer units of 6-18 carbon atoms (Laycock et al., 2014). PHAs are a family of isotactic polymers (Palmeiro-Sánchez et al., 2013) optically active with monomer units with just the R-configuration (Guérin et al., 2010). The obtained biopolymers are non-toxic, hydrophobic and water-insoluble as well as inert and stable in air. PHAs are well-known for having thermoplastic and/or elastomeric properties (Laycock et al., 2014). The most common PHAs are poly(3-hydroxybutyrate) (P3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)), which have similar properties to polypropylene and polyethylene, respectively, but with lower elongation at break and fragility (Laycock et al., 2014).

PHAs have variable properties depending on the type of structure adopted by the compound and this is dependent on the existing monomers and their layout. The copolymer P3HB is the most well-known PHA. Over 150 different hydroxyalkanoic acids have been reported (Sudesh et al., 2000). A comparison of the properties of the PHAs is provided in Table 1.2 (Anderson and Dawes, 1990; Khanna and Srivastava, 2005; Lee, 1996; Sudesh et al., 2000).

Table 1.2. Comparison of different PHA polymer properties (adapted from Anderson and Dawes (1990); Khanna and Srivastava (2005); Lee (1996); and Sudesh et al. (2000)(Harding et al., 2007)).

Polymer	Melting temperature (°C)	Young's Modulus (GPa)	Tensile Strength (MPa)	Elongation to break (%)	Impact strength (J/m)	Glass transition temperature (°C)
P(3HB)	179	3.5	40	5-680	50	4
P(3HB-co-3HV)						
3%mol 3HV	170	2.9	38	-	60	-
9%mol 3HV	162	1.9	37	-	95	-
14%mol 3HV	150	1.5	35	-	120	-
20%mol 3HV	145	1.2	32	-	200	-
25%mol 3HV	137	0.7	30	-	400	-
P(3HB-co-4HB)						
3%mol 4HV	166	-	28	45	-	-
10%mol 4HV	159	-	24	242	-	-
16%mol 4HV	-	-	26	444	-	-
64%mol 4HV	50	30	17	591	-	-
90%mol 4HV	50	100	65	1080	-	-
P(4HB)	53	149	104	1000	-	-

(-): Data not available

P(3HB-co-4HV): Poly(3-hydroxybutyrate-co-4-hydroxyvalerate)

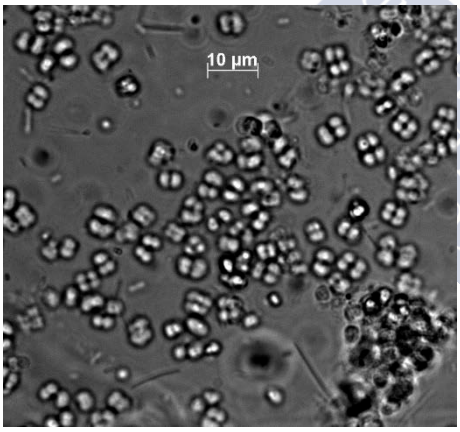
P(4HB): Poly(4-hydroxybutyrate)

Only the homopolymer of PHB; copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate; and 4-hydroxybutyrate and 3-hydroxyhexanoate have been industrially produced (Dias et al., 2006). It can be seen in Table 1.2 that a wide range of thermoplastics can be design using PHAs. For example, P(3HB) has been usually compared to polypropylene since they both have similar properties. For example,

P(3HB) has a tensile strength of about 40 MPa while polypropylene has a value of 34.5 MPa. The impact strength is also very similar with values of 50 and 45 J/m for P(3HB) and polypropylene, respectively. Nevertheless, parameters like the elongation to break might differ since P(3HB) has values in the range of 5 - 680 % while polypropylene has a value about 400 % (Harding et al., 2007).

1.4.5 PHA Production and Applications

Due to the diversity of hydroxyalkanoic acids composition and properties, PHAs are useful for several applications such as packaging, fabricating such despair products as medical and construction materials, etc. (Figure 1.10) with the advantage of being easily biodegradable. Up to date, commercial existing processes are based on pure cultures (Chen, 2010a) but these processes are still very expensive to outcompete the conventional plastics production from petroleum. Commercial PHAs are produced with many different pure cultures like recombinant *Escherichia coli* or *Alcaligenes latus* (Lemos et al., 2006; Salehizadeh and Van Loosdrecht, 2004). The PHA production capacity for strains like *Cupriavidus necator* or the aforementioned *Escherichia coli* can reach 80-90% in dry weight (Dias et al., 2006).



- Packaging
- Heat sensitive adhesives
- Latex
- Intermediates for fine chemicals
- Biofuels
- Bio-implant materials
- Drug delivery carriers
- Oligo-HA as nutrition supplements
- Other applications

Figure 1.10. Applications of PHAs.

Currently the most important industrial applications of PHAs are related to biomedicine. The most outstanding applications in this field are prosthesis and orthopaedic utilisation and also as drug delivery carriers because of its biocompatibility and biodegradability (Grage et al., 2009). Nevertheless, important brands like Wella (Germany), Procter and Gamble (US) and several other companies

used PHA as raw material to fabricate shampoo bottles and other things as packaging materials, shopping bags, feminine hygiene products, surgical material, etc (Anderson and Dawes, 1990; Chen, 2010a). Other utility found was the application of PHAs as biofuels (Zhang et al., 2009).

The first company in commercialising PHAs was Metabolix (USA). Several companies produce PHA nowadays at industrial scale (Table 1.3) (Chen, 2010a). However, at the present time, PHA production at industrial scale costs 4 to 9 times more than conventional plastics production (Moita and Lemos, 2012) and the average cost of PHA production with pure cultures is estimated in 8 €/kg of PHA (Reis et al., 2003). Due to this, PHA manufacture at industrial scale is still scarce compared to conventional plastics production and even to other biodegradable polymers (Braunegg et al., 1998).

Table 1.3. Commercialised PHAs produced from pure cultures and costly substrates.

Company	Type of PHA
ICI (UK)	PHBV
Chemie Linz (Austria)	PHB
BTF (Austria)	PHB
Biomers (Germany)	PHB, PHBV
BASF (Germany)	PHB, PHBV
Metabolix (US)	Several PHA
Monsanto (US)	PHB, PHBV
Meredian (US)	Several PHA
Kaneka (Japan)	Several PHA
Mitsubishi (Japan)	PHB
Biocycles (Brazil)	PHB
Bio-On (Italy)	Several PHA
ZheijansTian An (China)	PHBV
Yikeman Shandong (China)	Several PHA
Tianjin Green Bioscience (China)	P3HB-co-4HB

For the design of a competitive process for bioplastics production, the whole production development must be optimised but also new pathways should be explored. Hence, it would be of interest the use of microbial mixed communities since its use lowers the costs attributable to the sterile conditions and adding the benefit of the use of wastes as substrate (Reis et al., 2003; Serafim et al., 2008).

1.5 SALINE WASTES

The Executive Agency for Small and Medium Sized Enterprises (EASME) on behalf of the European Commission has been designed to manage several programs of the European Union regarding energy, environment and maritime fields. The interest of these spheres relies on the fact that enterprises and companies related to sea and coastal activities (shipyards, transport, energy, tourism, biotechnology, aquaculture…) are key drivers for the economic growth and progress of the European Union. All these sectors about economic activities related to the oceanic and maritime ambiances define the "*Blue Economy*" (European Commission-Executive Agency for Small and Medium-sized Enterprises, 2016).

Activities related to sea and maritime activities are of key importance in Galicia. In this North-Western Spanish Region in particular, aquaculture and fish-canning industries have a strong influence and importance in the local economy. Moreover, they are important for the wealth of the region but also at national levels, since nearly the total of the Spanish fish-canning industries is located in Galicia.

The Spanish National Association of Fish-Canning Manufacturers (Asociación Nacional de Fabricantes de Conservas de Pescado-ANFACO) states that there are 67 companies dedicated to the manufacturing of fish-canned products in Galicia. This represents the 85% of the economic value of the whole national production of fish-canned products and the 87% of the total volume. A number of 47 out of 67 companies are focussed on tuna products, which represents the 70% of the tuna-canned products in Spain (ANFACO-CECOPESCA, 2014).

Besides the economic benefits and the generation of employments, these industries generate large amounts of waste effluents that need to be treated. These waste effluents coming from fish-canning or aquaculture industries, in addition to high saline concentrations, are also characterised by abundant flows with high organic loads (Gebauer, 2004; Soto et al., 1990a). For example, an average fish-canning industry from Galicia, like FRINSA del Noroeste, S.A., discharges nearly

615,000 m^3/year of wastewaters with equivalent values of released organic matter and nutrients of 64.3 Tons N/year, 6.43 Tons P/year, and 509 Tons C/year just in 2014 (Spanish Department of Fishing and Agriculture and Food and Environment, 2017).

Particularly, tuna processing effluents have the highest organic loads and protein content among the diverse effluents of a fish-canning factory (Artiga et al., 2008). Moreover, fish-canning wastewaters also experience variations in terms of NaCl concentration, due to the seasonality of the product, the location of the plant, the type of fish treated and the process itself. For example, on a seaside factory, the octopus boiling wastewater contains 1.33 g NaCl/g COD while the mussel boiling wastewater has 2.24 g NaCl/g COD, and the fish flour line wastewater has just 0.23 g NaCl/g COD (Soto et al., 1990b). All these products can be processed in the same plant at different moments of the year, affecting the wastewater composition.

Physico-chemical processes were commonly used to treat saline waste effluents though the operational costs are particularly high due to the price of the required chemicals and the subsequent waste management. The most commonly used physico-chemical methods are sedimentation, flotation, filtration, precipitation, evaporation, coagulation-flocculation and membrane systems (Lefebvre and Moletta, 2006; Omil, 2003).

Biological systems, comprising anaerobic or aerobic biological systems, have been proved to be useful for the treatment of saline wastewater (Palmeiro-Sánchez et al., 2013). On the one hand, aerobic systems have high aeration costs and generate great amounts of sludge while, on the other hand, anaerobic systems generate less sludge volumes and have higher flexibility (Omil, 2003). In any case, the operation depends on the biomass adaptation, among other factors, but further investigation is needed about the physico-chemical and biological treatment of saline industrial effluents, or even the combination of both, so as to meet the regulations (Lefebvre and Moletta, 2006).

These saline effluents are eligible candidates for different valorisation methods:

- Biogas production from aerobic sludge produced in aerobic conditions in the secondary treatment.
- Obtaining of VFAs from high organic loaded saline wastewater.

- Production of PHAs by using acidifying high organic loaded saline wastewater.

As indicated in the previous sections, in the case of biogas (Section 1.2.3) and fatty acids production (Section 1.3.2), it is known that salinity affects the efficiency of the anaerobic digestion process (Lefebvre and Moletta, 2006). Another important factor that has to be taken into account in anaerobic environments is the toxicity of sulphide (Chen et al., 2008; Isa et al., 1986). In the case of the aerobic reactors for PHA production, the presence of sodium chloride affects the biomass accumulating activity (Section 1.4.3). Some waste streams, like the ones generated by the fish-canning industries, may contain significant amounts of NaCl or even transient concentrations of this salt (Mendez et al., 1995) and salinity has to be taken into account when this type of wastewaters are used as substrate for PHA production (Palmeiro-Sánchez et al., 2016a).

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Chapter 2
MATERIALS AND METHODS

SUMMARY

This chapter aims to describe the analytical methods used during the experimental work performed in this thesis. A brief description of the conventional parameters used for the liquid, gas and solid phases characterization is presented.

Some of the measured conventional parameters in the liquid phase-such as chemical and biological oxygen demand (COD and BOD), volatile fatty acids (VFAs), total and volatile suspended solid (TSS and VSS) and ammonium (NH_4^+)- were determined following *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). Sulphate, sodium and several inorganic anions and cations have been measured by analytical procedures optimised in our laboratories. The biomass, solid phase, was characterised by means of digital image analysis and microscopy for its identification and quantification. The content of polyhydroxyalkanoates (PHAs) was quantified by gas chromatography. The procedure to determine the biomass activity is also presented in this chapter. The composition of the gas phase was analysed by gas chromatography.

All the procedures are described in detail throughout this chapter. The specific analytical methods or the calculations used in a single part of the work and the corresponding experimental setups are described in the corresponding chapters.

A description of the mass balances performed for the different chapters of this thesis is also provided together with the calculations used for the corresponding kinetic and stoichiometric parameters.

Outline

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2.1 ANALYSIS OF THE LIQUID PHASE

This section describes the methods used for the determination of the conventional parameters measured in the liquid phase. Samples from the reactors were filtered through a mixed cellulose ester filter of 0.45 μm pore size (Advantec, Japan) so as to remove suspended solids for soluble fraction analysis.

2.1.1 Chemical Oxygen Demand (COD)

The Chemical Oxygen Demand (COD) is defined as the amount of oxygen required to oxidise the organic matter by the use of a strong chemical oxidant (potassium dichromate) in an acid medium. A catalyst (silver sulphate) is used to improve the oxidation of some organic compounds. After digestion, the remaining amount of unreduced $K_2Cr_2O_7$ is titrated with ferrous ammonium sulphate to determine the amount of $K_2Cr_2O_7$ consumed, being the amount of oxidised organic matter calculated in terms of oxygen equivalents. The main reaction is:

$$Cr_2O_7^{2-} + \text{Organic Matter} + H^+ \Leftrightarrow Cr_3^+ + CO_2 + H_2O$$

The total and soluble Chemical Oxygen Demand concentrations (COD_t and COD_s, respectively) are determined following the method 5220C of the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005) combined with another method for samples with high concentration of salts (Soto et al., 1989). The COD_t is determined using the raw sample, while filtered samples are used to determine COD_s.

Reagents

- Standard potassium dichromate digestion solution: 10.216 g of $K_2Cr_2O_7$ and 33 g of $HgSO_4$ are dissolved in 500 mL of distilled water. Then, 167 mL of concentrated H_2SO_4 are added. Then, the solution is cooled at room temperature and, finally, diluted to 1000 mL.
- Sulphuric acid reagent: 10.7 g of Ag_2SO_4 are added to 1 L of concentrated H_2SO_4. The solution can be used after 48 hours.
- Ferroin indicator solution: 1.485 g of $C_{18}H_8N_2 \cdot H_2O$ (phenanthroline monohydrate) and 0.695 g of $SO_4Fe \cdot 7\ H_2O$ are dissolved in 100 mL of distilled water.

- Potassium dichromate solution 0.05 N: An amount of 1.226 g of $K_2Cr_2O_7$, previously dried at 105 °C for 2 hours, is dissolved in 500 mL of distilled water.
- Ferrous ammonium sulphate titrant (FAS) 0.035 N: 13.72 g of $Fe(NH_4)_2(SO_4)_2{\cdot}6\ H_2O$ are dissolved in distilled water. Then, 20 mL of concentrated H_2SO_4 are added and, finally, the solution is cooled and diluted to 1000 mL.

Determination procedure

This procedure is applicable to samples with COD concentrations between 90 and 900 mg/L. The preparation of the samples goes as follows: 2.5 mL of sample are placed in a 10 mL Pyrex® glass tube. An amount of 1.5 mL of digestion solution is added, together with 3.5 mL of sulphuric acid reagent. This last reagent must be gently poured down the wall of the tube, which has to be rather leaned to avoid the mixing at this point. A blank sample using distilled water is prepared in the same way. This blank acts as "reference" value.

The Pyrex® glass tubes are tightly sealed with Teflon® and covered with Bakelite® caps so as to avoid the leakage of produced gases. After this, the tubes are shaken, until the complete mixing, and placed for 2 h in the block thermo-digester (HACH Lange 16500-100, USA) preheated at 150 °C. After the digestion period, the Pyrex® tubes are cooled at room temperature. Finally, the content of the tubes is transferred into a stirred beaker, together with 3 drops of ferroin indicator, and titrated with standard FAS. The final titration point is indicated by a sharp change of colour from turquoise to reddish brown.

The FAS solution is standardised daily following the subsequent procedure: 5 mL of distilled water are added into a small beaker together with 3.5 mL of sulphuric acid reagent. The liquid must be cooled at room temperature before the addition of 5 mL of the standard potassium dichromate solution (0.05 N). Approximately 1-2 drops of ferroin must be added as indicator before titrating with FAS in a similar way as applied to the samples. The molarity of the FAS solution is calculated using the Equation 2.1:

$$M_{fas} = \frac{5 \cdot 0.05}{V_{fas}} \qquad \text{Equation 2.1}$$

where:

M_{fas}: FAS molarity (mol/L)

V_{fas}: volume of FAS consumed in the titration (mL)

The COD is calculated following Equation 2.2:

$$\text{COD [mg/L]} = \frac{(A - B) \cdot M_{fas} \cdot 8000}{V} \qquad \text{Equation 2.2}$$

where:

A: volume of FAS solution consumed by the blank (mL)

B: volume of FAS solution consumed by the sample (mL)

8000: milliequivalent weight of oxygen multiplied by 1000 (mL/L)

Interferences

The most common interference of this method is the chloride ion at concentrations higher than 2 g/L of Cl^-. Chloride reacts with silver ion to precipitate silver chloride, and thus inhibits the catalytic activity of silver. Bromide and Iodide can interfere similarly.

2.1.2 Biological Oxygen Demand (BOD)

The biological oxygen demand (BOD) is a parameter that measures the biodegradability of the organic matter in a liquid sample. It allows the estimation of the dissolved oxygen used by the microorganisms to oxidize the organic matter present in a liquid sample. The reaction can be described as follows:

$$O_2 + \text{Organic Matter} \xrightarrow{\text{microorganisms}} \text{microorganisms} + CO_2 + H_2O$$

In the present research work, the measurement was done on day 5 of the experiment (BOD_5).

The equipment to determine the BOD_5 is an *Oxitop* (WTW, Germany). This system is based on the difference of pressure generated due to the CO_2 production. The measurement is done by determining the pressure with a piezoresistive

electronic sensor, which directly gives the BOD_5 information in mg/L. This system acquires and saves data during 5 days.

Reagents

- Solutions of NaOH or HCl to adjust the pH.
- NaOH lentils to adsorb the generated CO_2.
- Nitrification inhibitor (commercial solution of NTH600).

Determination procedure

The samples for BOD_5 can degrade very fast so it is necessary to preserve them at low temperature (4 °C) while they are not analysed. Samples should be as fresh as possible. The pH of the sample must be within 6.5 and 8.3. Small amounts of HCl or NaOH solutions should be added in case the pH value is higher or lower, but taking care that the supplied amount of acid or base represents less than 0.5% of the analysed sample.

Having this in mind, a known volume of sample must be introduced in the incubation bottles. The volume of sample must be estimated as a function of the expected BOD_5 (Table 2.1). If a high pollution degree is estimated, then the dissolved oxygen consumption will be very high and it will surpass the system saturation capacity (9.17 mg O_2/L, at 20 °C). Then, the dilution of the sample with distilled water will be necessary according to Table 2.1. The expected BOD_5 can be inferred from the COD value, which just takes 3 hours of determination. For a rough estimation, it can be supposed that BOD_5 would be about 80% of the COD.

Then, a nitrification inhibitor NTH600 must be added in the amount indicated in Table 2.1. Finally, it is important to take into account that some wastewater samples do not have a significant microbial population -for example, disinfected residues or residues with high or low pH values- so it is necessary to inoculate the incubation bottles with approximately 1 mL of an activated sludge sample.

After all this sample preparation, a rubber cap with a couple of lentils of NaOH is located before the placement of the Oxitop device to adsorb the generated CO_2. The incubation bottles are located in a thermostatic chamber at 20 °C for 5 days.

Table 2.1. Preparation of the sample for BOD_5 determination.

Sample volume (mL)	Measurement range (mL/L)	Oxitop factor	Drops of NTH600
432	0-40	1	9
365	0-80	2	7
250	0-200	5	5
164	0-400	10	3
97	0-800	20	2
43.5	0-2000	50	1
22.7	0-4000	100	1

The BOD_5 is calculated in the following Equation 2.3:

$$BOD_5 = L_5 \cdot F_0 \cdot F_d \qquad \text{Equation 2.3}$$

where:

BOD_5: Biological Oxygen Demand concentration after 5 days (mg /L)

L_5: value indicated by the *Oxitop* device after 5 days (mg O_2/L)

F_0: *Oxitop* factor (indicated in Table 2.1)

F_d: dilution factor

2.1.3 Volatile fatty acids (VFAs)

Volatile fatty acids (VFAs) are fatty acids with a carbon chain of six carbons or fewer such as acetic (HAc); propionic (HPr); i-butyric and n-butyric (HBu); and valeric (HVa), which are intermediate products of the anaerobic digestion. The measurement of the VFA concentration is commonly used as a control test for anaerobic digestion since the accumulation of VFA reflects a kinetic disequilibrium between the activity of the acid producers and the acid consumers (Switzenbaum et al., 1990) and it is an indicator of the anaerobic process destabilization. The monitoring of the VFA concentration is also used to observe the feasting by the PHA-producing organisms so as to calculate de consumption rates and the mass balances.

VFAs are determined following a similar method to the one described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005).

Reagents

- Pivalic acid Standard: This standard is prepared as a solution of pivalic acid (0.2 vol%) and formic acid (8 vol%) in demi-water.

Determination procedure

The VFA tests are prepared from the filtered sample, diluted with distilled water if needed. The sample for the analysis is constituted by 50 vol% of sample and 50 vol% of pivalic acid as internal standard.

After this preparation, VFAs are determined by gas chromatography (GC) (Hewlett Packard 5890A, USA), equipped with a flame ionization detector (FID) and an automatic injector (Hewlett Packard 7673A, USA). The determination is performed using a glass column (3 m long and 2 mm of internal diameter) filled with Chromosorb WAW (mesh 100/120) impregnated with NPGA (25%) and H_3PO_4 (2%). The column, injector and detector temperatures are 105, 261 and 281 °C, respectively. Gas N_2, previously saturated with formic acid before entering the injector, is used as carrier gas at a flow of 24 mL/min. Air and H_2 are used as auxiliary gases with flows of 400 and 30 mL/min, respectively. The VFA, after being separated in the column according to their molecular weights, are burnt in a H_2-air flame and finally measured in the flame ionisation detector (FID) at 281 °C.

The quantification of the sample is made with a calibration curve for each acid in the range of concentrations of 0-1.2 g/L, using pivalic acid as internal standard.

Calibration curve

Firstly, a concentrated VFA stock solution is prepared by mixing 50 mL of distilled water with 0.2 mL of acetic, 0.2 mL of propionic, 0.2 mL of n-butyric, 0.1 mL of i-valeric and 0.1 mL of n-valeric. Secondly, a diluted VFA stock solution is prepared by mixing 10 mL of the concentrated solution together with 25 mL of distilled water. From this second stock solution, diluted samples are prepared by applying dilutions with demi-water from 1:1 to 1:25.

The obtained chromatogram gives information about the area of the obtained peaks corresponding to each analysed VFA. Each VFA is identified by the retention time of its corresponding peak. Finally, the area is related with the VFAs concentration (g/L) by means of the calibration curves, like those shown in the figure below (Figure 2.1):

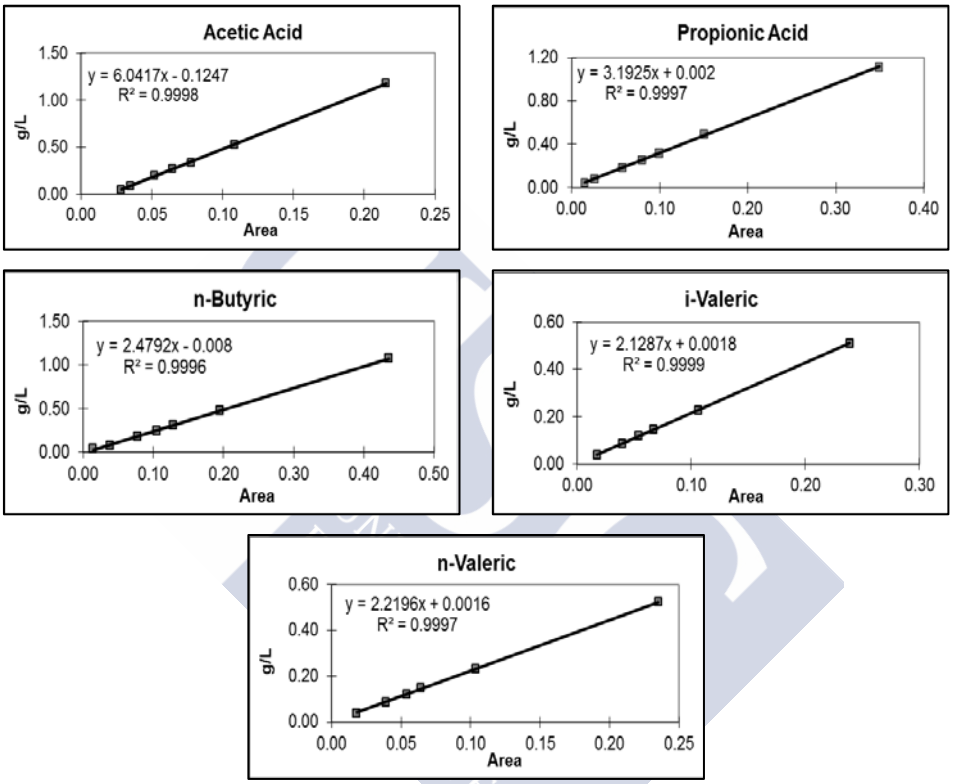

Figure 2.1. Calibration curves for the determination of the different VFAs.

where:

Area: is the ratio between the VFA area and the pivalic internal standard area

x: Area

y: VFA concentration (g/L)

2.1.4 Ammonium (NH_4^+)

Total ammonium (NH_4^+) was determined spectrophotometrically by a method in which indophenol blue was produced by the reaction of ammonia with salicylate and hypochlorite, in the presence of sodium nitroprusside (Bower and Holm-Hansen, 1980). This method uses compounds less toxic than the phenol–hypochlorite method described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). The characteristic blue colour produced by the concentration of ammonia make the assay useful for the direct, visual estimation of ammonia in culture systems. The liquid sample must be filtered prior to its analysis.

Reagents

- Reagent A: This solution is prepared by mixing 0.28 g/L of sodium nitroprusside and 440 g/L of sodium salicylate in distilled water.
- Reagent B: This solution is prepared by mixing 18.5 g/L of NaOH and 120 g/L of sodium citrate in distilled water.
- Reagent C: This is a commercial solution of sodium hypochlorite.
- Reagent D: This solution is prepared by mixing 7 parts of reagent B and 1 part of reagent C. Reagent D is stable for 1 hour after preparation.

Determination Procedure

It starts with the addition of 600 µL of reagent A and 1 mL of reagent D to 5 mL of filtered sample. Reaction time is fixed between 2 and 3 hours. The samples must be protected from light. The measurement of the coloured sample is done with a spectrophotometer (CE 7200, Cecil Instruments, UK) at a wavelength of 640 nm. The concentration is given by comparison of the obtained absorbance with the values of the calibration curve (Figure 2.2).

Calibration curve

Firstly, a stock solution of 10 mg NH_4^+/L is prepared. Secondly, more diluted samples are prepared so as to generate a reliable calibration curve. The spectrometer gives information about the absorbance of each sample, which is related to the NH_4^+ concentration (mg NH_4^+-N/L) by means of the calibration curve (Figure 2.2).

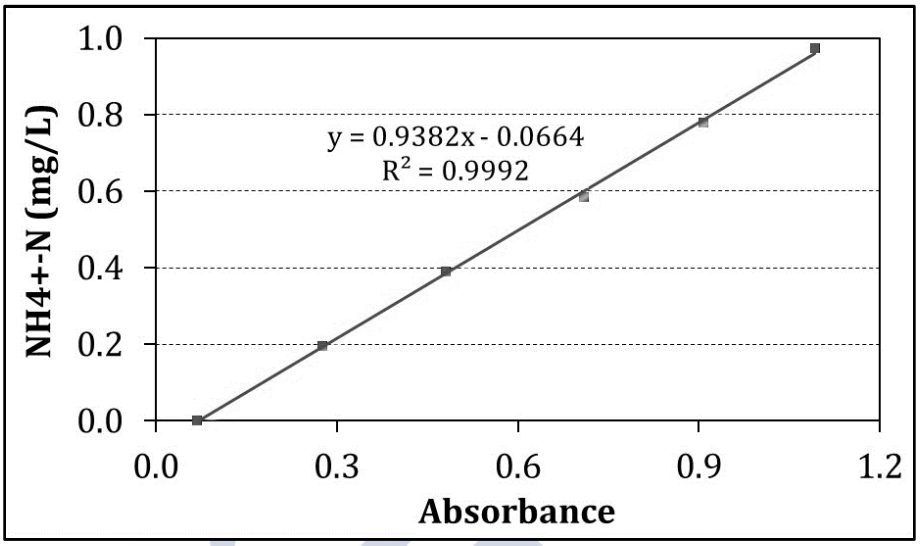

Figure 2.2. Calibration curve for ammonium determination.

where:

x: Absorbance of the sample

y: NH_4^+-N concentration (mg/L)

2.1.5 Alkalinity

The alkalinity is an indicator of the acid neutralizing capacity of a wastewater sample (Speece, 1996). Alkalinity measurements are used in the control of the degree of acidification and stability during the anaerobic digestion process.

Alkalinity is related to the presence of buffering agents like salts and weak acids. At pH close to neutrality, the most common buffer substances are carbonate, bicarbonate and hydroxides, although it may also include contributions from borates, phosphates, silicates and/or other bases. Alkalinity thus depends on the end-point pH used. The pH values are suggested as the equivalence points for the corresponding alkalinity concentration as milligrams of $CaCO_3$ per litre. The total alkalinity (TA) is determined at pH 4.3. It can be considered as the sum of the alkalinity due to bicarbonate together with that corresponding to the VFAs in units of mg/L in equivalents of $CaCO_3$. The partial alkalinity (PA), with an end-point at pH 5.75, corresponds to the alkalinity due to the presence of bicarbonate (Jenkins et al.,

1983). The intermediate alkalinity (IA) is defined as the difference between TA and PA, and corresponds approximately to the alkalinity related to the VFA content (Ripley et al., 1986). Various authors established that the relation between IA and TA is an adequate parameter for the evaluation of the stability of the anaerobic digestion process (Soto et al., 1993; Wentzel et al., 1994). It should not exceed the value of 0.3 just to avoid the accumulation of VFAs in the system.

Reagents

- Solution of the standard acid (H_2SO_4): the concentration of this solution varies (0.05-0.5 N) in relationship with the amount of alkalinity expected.

Determination procedure

The alkalinity was determined following the method described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). It consists in the titration of a sample volume (normally 25 mL) at room temperature with a standard acid (H_2SO_4 standardized against Na_2CO_3) to reach the desired pH (pH 5.75 for PA and pH 4.3 for TA). The alkalinity value (mg $CaCO_3$/L) is calculated from the equation (Equation 2.4):

$$\text{Alkalinity} = \frac{A \cdot N \cdot 50000}{V} \qquad \text{Equation 2.4}$$

where:

A: is the volume of standard acid (usually H_2SO_4) used to decrease the pH to 5.75 (PA) or to 4.3 (TA) (mL).

N: is the normality of the standard acid (usually H_2SO_4) in equivalents/L. This concentration depends on the expected alkalinity of the water and it usually varies from 0.05 to 0.1 N.

V: is the sample volume (mL). It usually is approximately 25 mL.

2.1.6 Measurement of anions and cations

The measured anions are: Cl^-, Br^-, NO_3^-, NO_2^-, SO_4^{2-} and the measured cations are: Li^+, Na^+, K^+, NH_4^+, Ca^{+2}, Mg^{+2}.

The samples are filtered through a mixed cellulose ester filter of 0.45 μm pore size (Advantec, Japan) prior to its analysis and stored in a freezer if it is not going to be immediately analysed. A minimum of 10 mL is needed.

Ions are measured in the samples by ion chromatography (IC) using a 861 Advanced Compact IC with and 838 Advanced Sample Processor (Metrohm, Switzerland). The analysed sample goes through the column and the ions are separated by retention along the resin column (Metrosep C3-250, Metrohm, Switzerland). Each ion leaves the column at a characteristic retention time.

Then, the sample passes through a condutimetric detector after the separation, where the obtained signal corresponding to each retention time is registered. The resulting chromatograms identify each measured ion by its retention time position together with the amount of ion, which is related to the area.

Reagents for anions

- Sulphuric acid (H_2SO_4) 50 mM.
- Acetonitrile (CH_3CN) 10%.
- Standard solutions of each anion (Cl^-, Br^-, NO_3^-, NO_2^-, SO_4^{2-}) are prepared (see Table 2.2.).
- A volume of 2 L of eluent is prepared with concentrations of 3.2 mM of Na_2CO_3 and 1.0 mM of $NaHCO_3$.

Reagents for cations

- Standard solutions of cations (Li^+, Na^+, K^+, NH_4^+, Ca^{+2}, Mg^{+2}) are prepared (see Table 2.3.).
- A volume of 2 L of eluent of Nitric acid (HNO_3) 3.5 mM.

Calibration

The chromatogram gives information about the ion type and its quantity by means of a calibration procedure. The final concentrations of the detected ions are automatically determined by the equipment and its software, giving the final result in units of mg/L.

Table 2.2. Calibration data for anions.

IC Calibration - Anions					
Dilution of the Standards	**Level 1** 1:1	**Level 2** 1:2	**Level 3** 1:10	**Level 4** 1:50	**Level 5** 1:100
Anion	**Concentration (mg/L)**				
Cl^-	100	50	10	2	1
NO_2^-	5	2.5	0.5	0.1	0.05
Br^-	20	10	2	0.4	0.2
NO_3^-	50	25	5	1	0.5
SO_4^{2-}	150	75	15	3	1.5

Table 2.3. Calibration data for cations.

IC Calibration - Cations					
Dilution of the Standards	**Level 1** 1:1	**Level 2** 1:2	**Level 3** 1:10	**Level 4** 1:50	**Level 5** 1:100
Anion	**Concentration (mg/L)**				
Li^+	5	2.5	0.5	0.1	0.05
Na^+	150	75	15	3	1.5
NH_4^+	10	5	1	0.2	0.1
K^+	50	25	5	1	0.5
Mg^{2+}	50	25	5	1	0.5
Ca^{2+}	50	25	5	1	0.5

2.1.7 Other control parameters

2.1.7.1 pH

The pH was measured with an electrode (52-03, Crison Instruments, USA) equipped with an automatic compensatory temperature device (21-910-01, Crison Instruments, USA) and connected to a digital meter (pH). The sensitivity of the instrument was 0.01 pH units. The electrode was calibrated at room temperature with two standard buffer solutions of pH 7.02 and 4.00.

2.1.7.2 Dissolved oxygen (DO)

Dissolved oxygen (DO) was measured using a digital multimeter device (HQ40D, Hach Lange, USA) equipped with a Luminescence-based DO probe (Intellical LDO, Hach Lange, USA). These devices do not need calibration since it is digitally done by the distributor of the equipment.

2.1.7.3 Temperature

In the aerobic reactors, the oxygen probes mentioned in the previous section were equipped with a thermopar that measured the temperature. In the anaerobic reactor, the temperature was also measured with a digital thermometer so as to control the performance of the thermostatic bath.

2.2 ANALYSIS OF THE GAS PHASE

In this section, the methods used for the determination of several parameters in the gas phase are described.

2.2.1 Biogas composition

The biogas composition (N_2, CH_4, CO_2 and H_2S) is determined by Gas Chromatography (GC) (HP-5890 Series II, USA) equipped with a Thermal Conductivity Detector (TCD). The stainless-steel column has an external diameter of 1/8" and is 2 m long. It is filled with Porapack Q80/100 (Supelco, USA). The temperatures of the injector, column and detector are 110, 35 and 110 °C, respectively. The oven temperature was fixed at 35 °C during 45 minutes, then, it was increased up to 100 °C at a rate of 70 °C/min. Helium was used as carrier gas with a flow of 46 mL/min. The sample volume (1 mL) was injected through a septum into the entrance of the instrument.

The calibration is performed with a standard mixture of gases (CH_4: 66%; CO_2: 30%; N_2: 2% and H_2S: 2%) by means of a response factor method, using the CO_2 as internal standard. The equipment is calibrated periodically and the equation relates the area with the percentage of each gas in the biogas extracted from the reactor by means of factors for each compound (f_{CH4}: 0.65; f_{CO2}: 1; f_{N2}: 1.05, and f_{H2S}: 0.99)). The sample of biogas is taken from the reactor with a syringe. Few seconds later it is directly injected into the chromatograph. The chromatograms given by the

equipment provide information about the area of each one of the identified gases. The percentage of each gas is calculated by the following equations:

$$N_2(\%) = \frac{A_{N2}/f_{N2}}{A_{N2}/f_{N2} + A_{CH4}/f_{CH4} + A_{CO2}/f_{CO2} + A_{H2S}/f_{H2S}} \quad \text{Equation 2.5}$$

$$CH_4(\%) = \frac{A_{CH4}/f_{CH4}}{A_{N2}/f_{N2} + A_{CH4}/f_{CH4} + A_{CO2}/f_{CO2} + A_{H2S}/f_{H2S}} \quad \text{Equation 2.6}$$

$$CO_2(\%) = \frac{A_{CO2}/f_{CO2}}{A_{N2}/f_{N2} + A_{CH4}/f_{CH4} + A_{CO2}/f_{CO2} + A_{H2S}/f_{H2S}} \quad \text{Equation 2.7}$$

$$H_2S(\%) = \frac{A_{H2S}/f_{H2S}}{A_{N2}/f_{N2} + A_{CH4}/f_{CH4} + A_{CO2}/f_{CO2} + A_{H2S}/f_{H2S}} \quad \text{Equation 2.8}$$

2.2.2 Biogas production

The measurement of the methane production (MP) in the continuous anaerobic digester is performed using a flow-meter designed by Veiga et al. (1990) (Figure 2.3). It consists in two glass columns with a height of 20 cm and an internal diameter of 3 cm.

Both columns are directly connected in their base by a tube of 1 cm of diameter. Their central regions are connected by a trap designed as a J-tube with an internal diameter of 0.5 cm, with one arm going from Column I towards the inside of Column II, as described in Figure 2.3.

The columns contain a liquid (usually water) which is displaced by the produced gas from the digester entering the device through the top of Column II. In Column I, two stainless-steel electrodes of different lengths are connected in series with an electromechanical pulse counter (Omron, Japan) that counts one unit (one pulse), every time the liquid in Column I goes up and connects the two electrodes. Once the maximum liquid level is reached, the water returns to Column II through the J-tube and liquid is at the same level in both columns. This movement of liquid is caused by the fact that the level of the liquid in Column II falls below the lower mouth of the J-tube and the liquid can return from Column I to Column II. The equipment must be calibrated to know the volume (mL) of liquid displaced in each pulse.

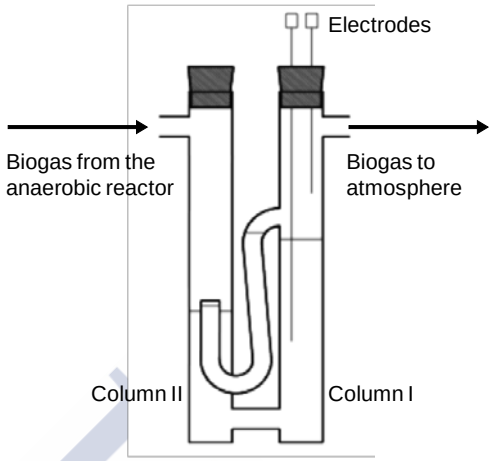

Figure 2.3. Device used for the measurement of the methane flow (Veiga et al., 1990)

2.3 BIOMASS CHARACTERISATION

In this section, the methods used for the determination of the conventional parameters measured in the solid phase are described.

2.3.1 Solids concentrations (TSS and VSS)

The total Suspended Solids (TSS) and the Volatile Suspended Solids (VSS) concentrations are determined according to the methods described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). The VSS concentration is assimilated to the content of biomass in the sample.

Determination procedure

For the determination of the TSS, a known volume of the sample is collected to obtain a residue between 2.5 and 200 mg.

Firstly, a fibre glass filter (Whatman, GF/C, 4.7 cm of diameter, 1.2 μm of pore size) is placed in an oven at 105 °C for half an hour to remove the humidity and get a constant weight. Then, it is placed inside a desiccator to achieve room temperature. Then it is weighed and the obtained weight is F_0.

The second step is filtering a known volume of sample (V_0) usually between 5 and 50 mL. The sample is well-mixed and filtered through the dried fibre glass filter of known weight. The residue retained on the filter is dried for at least 2 hours at 103-105 °C, until reaching a constant weight. Then, it is located inside a desiccator till it achieves room temperature. It is finally weighed and the weight obtained is F_1. The increase in the weight of the filter represents the TSS. The concentration of TSS is determined according to the following equation:

$$\text{TSS}\left({}^{g}/_{L}\right) = \frac{F_1 - F_0}{V_0} \qquad \text{Equation 2.9}$$

Finally, for the determination of the VSS, the filter previously dried to obtain the TSS concentration is burnt inside a muffle furnace at 550 °C for half an hour. Then it is located inside the desiccator to reach room temperature and then weighed. The obtained weight is F_2. The weight lost during ignition corresponds to the VSS content and its concentration is determined according to Equation 2.10:

$$\text{VSS}\left({}^{g}/_{L}\right) = \frac{F_1 - F_2}{V_0} \qquad \text{Equation 2.10}$$

Interferences

Highly mineralized water with a significant concentration of calcium, magnesium, chloride and/or sulphate may be hygroscopic and require prolonged drying, desiccation and rapid weighing. Some inorganic salts such as hydroxides, carbonates or ammonium salts are decomposed and volatilised at 550 °C and, therefore, it can give a higher value than the real one for the volatile content in the sample.

2.3.2 Polyhydroxyalkanoates (PHAs)

The determination of polyhydroxyalkanoates (PHAs) in the biomass samples is done by using an adaptation of a method of Smolders et al. (Smolders et al., 1994). This analytical method is used in the quantitative determination of the two main PHA monomers: polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV).

Reagents

- Digestion solution: a solution of 1 L constituted by a ratio of 4:1 of 1-propanol (99 vol%) and hydrochloric acid (36.5-38 vol%) is prepared. It must be stored in the fridge and preserved from light.
- 1,2-dichloromethane (HPLC quality)
- Formaldehyde (37-38 vol%)
- Commercial standard of PHBV (88:12 wt%)*
- Benzoic acid Standard is used as the internal standard. The concentration for this solution is 20 g/L. An amount of 50 mL of solution is prepared by adding 1 g of benzoic acid to 50 mL of 1-propanol. This solution must be preserved from light and stored in the fridge for a maximum of 12 months.
- Sodium sulphate anhydrous (99 wt%)

Determination procedure

Biomass samples are taken with a syringe and located in tubes. Approximately 3-5 drops of formaldehyde (37 vol%) are added to stop any biological activity. Then, the samples are immediately stored in a fridge for few hours and later they are centrifuged at 5000 rpm for 10 minutes. The tubes with the samples are covered with Parafilm® and stored in a freezer for, at least, 2 hours. The frozen samples are freeze-dried for about 24 hours, depending on the volume.

These stored samples are weighed in an analytical balance (Mettler Toledo, USA). Measured samples are located into Pyrex® glass tubes of 10 mL. The reagents are added as soon as all the samples are measured. An amount of 50 µL of the benzoic acid standard is added in each tube. The following step is the addition of 1.5 mL of the digestion solution and 1.5 mL of 1, 2-dicloromethane. To avoid any losses due to the volatility of the organic compounds, it is important to make sure that the bakelite caps are screwed down securely and the Pyrex® tubes are completely sealed. The Pyrex® glass tubes are placed in a thermo-reactor at 100 °C (Hach-Lange 16500-100, USA) for 180 minutes. During this time, the reactions for the PHA extraction occur. The hydrochloric acid breaks the cell walls and hydrolyses the PHB. The propanol produces the linear ester of the PHB. After the digestion, the samples are cooled to room temperature. Straight afterwards, 3 mL of distilled water are added and all the tubes are energetically shaken in a vortex (IKA Works INC., Germany) to separate the two liquid phases (organic and aqueous ones). The

samples are located in a tube-rack for at least 30 minutes to allow the appropriate separation of these two liquid phases. They can also be centrifuged at approximately 2500 rpm for 5 minutes in order to have a better and fastest separation. The PHB is present in the organic fraction.

A sample of about 1 mL is taken from the organic phase with a glass pipette and it must be filtrated and dried. The filtration and drying of the sample are done by assembling pipette tips of 1 mL as support and pushing inside a small amount of glass wool -which would act as a filter- topped with sodium sulphate anhydrous, which acts as the dryer agent.

Finally, the filtered and dried samples are quickly located into GC vials and properly sealed with aluminium caps provided with a septum as they are going to be analysed by GC (6850 Series II, Hewlett Packard, USA). This apparatus is equipped with a flame ionization detector (FID) with a mixture of H_2/air at 40:400 mL/min; an automatic injector (6850 Series II, Hewlett Packard, USA) which injects 1 µL of sample at 250 °C; and the software Chemstation®. The chromatographic column used for the PHA detection was an HP-INNOWAX 30 m x 0.25 mm x 0.25 µm (Agilent, USA). The carrier gas is Helium supplied at a constant velocity of 1 mL/min. The oven operates at a temperature ramp from 60 °C to 230 °C which increases at a velocity of 10 °C/min. The compounds are distinguished by the different retention times of each one. The amounts of PHB and PHV in the samples are calculated by comparing the areas of the peaks in the chromatograms with the values of the calibration curves obtained as described below.

Calibration curve

For the preparation of the calibration curves, a minimum of 5 different PHBV standard samples, of weights from 1 to 10 mg approximately, are prepared. Benzoic acid is used as internal standard. Then, these samples are processed just like biomass samples (described in the previous section, *Determination Procedure*). The calibration curve is calculated by plotting the weight of the sample against its corresponding ratio of area value. This ratio is calculated by dividing the area corresponding to the peak of PHA by the area of the peak of the benzoic acid used as internal standard. The coefficient of determination (R^2) for this curve is expected to be higher than 0.99.

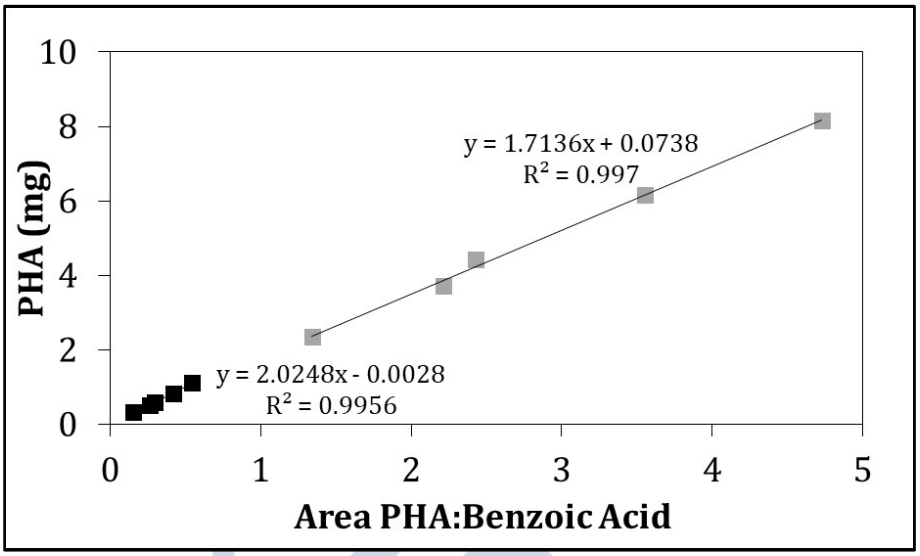

Figure 2.4. Calibration curves for PHB (■) and PHV (■) determination.

where:

x: is the value of the area of the ratio between the PHB or PHV area and the area of the benzoic acid internal standard solution

y: amount of PHA in the sample (mg)

Calculations

Samples for the extraction of biopolymers must be weighed accurately in order to calculate the percentage of PHA present in the sample. The final result is expressed in terms of wt% (mg_{PHA}/mg_{SAMPLE}). The following equation (Equation 2.11) is for calculating the percentage of PHA (wt% PHA) over the measured sample, expressed in dry weight.

$$\%PHA = \frac{W_{PHA}}{W_{SAMPLE}} \qquad \text{Equation 2.11}$$

where:

W_{PHA}: weight of the PHA calculated by means of the calibration curve (mg)

W_{SAMPLE}: weight of the sample (mg) measured as TS

2.3.3 Fats

Fats concentration is measured from biomass samples by solvent extraction. Fats are determined following the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). The samples must be treated at pH<2 and stored in the fridge if they are not going to be analysed immediately.

Reagents

- Petroleum ether

Determination procedure

The biomass sample is located in a separatory funnel together with 30 mL of petroleum ether. The mixture must be shaken vigorously for a couple of minutes. The narrow part of the funnel is located upwards so as to open the stopcock and release the produced gasses. It is important to remark that the volume of sample depends on its fats concentration and several assays must be attempted previously to obtain the value of the fats concentration.

After a good mixing of the sample and the petroleum ether, the separatory funnel must be located in a stand with a metal ring so as to allow the separation of the two phases. The organic phase is filtrated through a glass funnel with a Whatman® filter and poured into a distillation flask of known weight. Then, a distillation at 70 °C is performed with a rotary evaporator (Buchi, Switzerland). Finally, the flask is located into an oven at 105 °C for 2 hours and weighted after cooling. The amount of fats in the sample corresponds to the flask weight increase.

Calculations

Samples for the determination of fats must be weighed accurately in order to calculate the exact percentage present in the sample. The final result is expressed in terms of concentration (mg Fats/L Sample) by using Equation 2.12:

$$\text{Fats } (^{mg}/_{L}) = \frac{(P_2 - P_1) \cdot 1000}{V} \qquad \text{Equation 2.12}$$

where:

P_1: weight of the distillation flask (mg)

P_2: weight of the distillation flask after the evaporation (mg)

V: volume of the sample (mL)

2.3.4 Proteins

The determination of proteins is done according to a modified method based on other two methods (Frolund et al., 1995; Lowry et al., 1951). The procedure is based on two chemical reactions: the first is the Biuret reaction, in which the alkaline cupric tartrate reagent complexes the peptide bonds of the protein. This is followed by the reduction of the Folin and Ciocalteu's phenol reagent, which yields a purple colour. The absorbance of the resultant coloured solution is measured at a wavelength of 750 nm and the protein concentration is determined from the calibration curve done with a standard of bovine serum albumin (BSA).

Reagents*

- Solution A: 20 g Na_2CO_3 and 4 g NaOH are dissolved in 1 L of distilled water
- Solution B: 1 g $CuSO_4 \cdot 5\ H_2O$ is dissolved in 100 mL of distilled water
- Solution C: 2 g of sodium potassium tartrate are dissolved in 100 mL of distilled water
- Solution D: 50, 0.5 and 0.5 mL of solutions A, B and C, respectively, are mixed. This new solution must be prepared in the moment of the analysis, and it can only be stored for more than 8 hours
- Solution E (Folin and Ciocalteu's phenol reagent): 100 mL of commercial Folin and Ciocalteu's phenol reagent is dissolved in 100 mL of distilled water

*All solutions must be stored at 4 °C.

Determination procedure

A sample volume of 1.5 mL (diluted if necessary) is rapidly mixed with 2.1 mL of reagent D and left for 10 minutes at room temperature. Then, 0.3 mL of reagent E are added and mixed. After 45 minutes of reaction time, the absorbance is ready to be measured. A blank with the reagents and distilled water instead of the sample is also measured as reference.

Once the reaction takes places the samples are transferred to cuvettes and their absorbance is measured at a wavelength of 750 nm with a spectrophotometer (CE

7200, Cecil instruments, UK). The protein concentration is determined from a calibration curve, using as standard bovine serum albumin (BSA).

Calibration curve

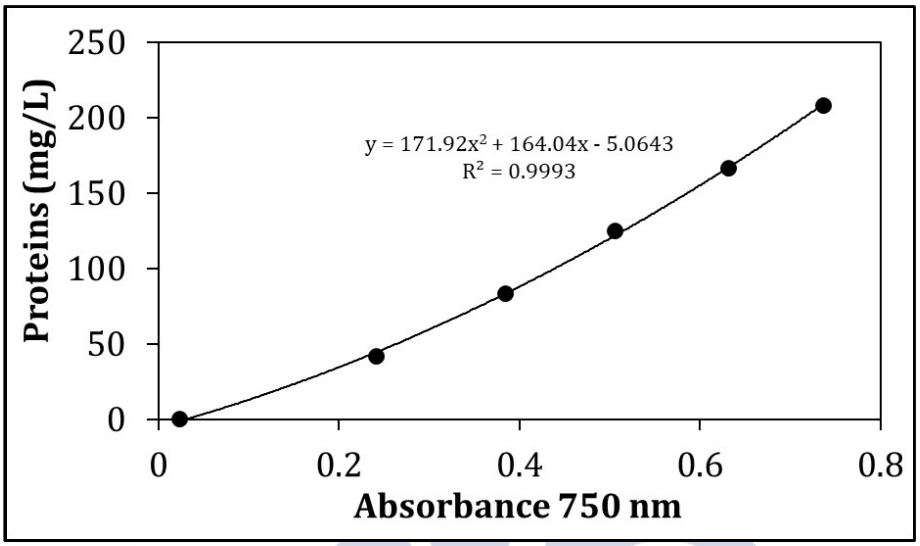

Figure 2.5. Calibration curve for proteins

where:

x: is the value of the absorbance of the sample measured a 750 nm

y: is the protein concentration (mg/L)

For the elaboration of the calibration curve, a solution of 1 g/L of BSA is prepared and standard samples are in the range of 0-250 mg/L. Then, successive dilutions are applied in order to prepare the standards for the calibration curve with concentrations of approximately 0, 40, 80, 150, 200 and 250 mg/L of BSA. The calibration curve is calculated by plotting the absorbance values of these BSA concentrations versus their corresponding protein concentrations (Figure 2.5).

2.3.5 Carbohydrates and sugars

The concentration of carbohydrates and sugars is analysed using a modified phenol–sulphuric acid method (DuBois et al., 1956). The analysed samples react with

phenol and sulphuric acid, giving to the liquid an orange colourization, which can be measured by spectrophotometry at 490 nm.

Reagents*

- Reagent A: Phenol solution at 5 vol%
- Reagent B: Sulphuric acid at 97 vol%

Determination procedure

A volume sample of 1 mL is rapidly mixed with 1 mL of reagent A and left for 10 minutes at room temperature. After this, 5 mL of reagent B are added and left for 5 minutes so as to cool down. Then, the mixture is vigorously agitated. After 25 minutes of reaction time, the absorbance is ready to be measured. A blank with the reagents only and distilled water instead of sample is measured as reference value.

The samples are transferred to quartz cuvettes and the absorbance is measured at a wavelength of 490 nm with a spectrophotometer (CE 7200, Cecil instruments, UK). The concentration of carbohydrates and sugars is determined from a calibration curve, using D-glucose monohydrate as standard.

Calibration curve

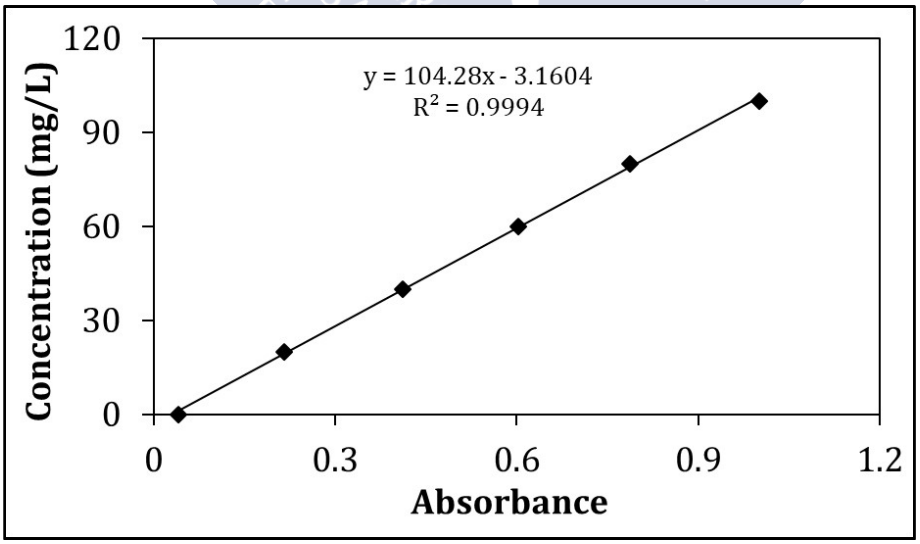

Figure 2.6. Calibration curve for sugars and carbohydrates

where:

x: is the value of the absorbance of the sample measured at 490 nm

y: is the carbohydrates and sugars concentration (mg/L)

For doing the calibration curve, a solution of 2 g/L of D-glucose monohydrate is prepared. Successive dilutions are done (0, 20, 40, 60, 80 and 100 mg/L of D-glucose monohydrate) to dilute the standard samples within the range of 0-100 mg/L. The calibration curve is calculated by plotting the absorbance values of the standards versus their corresponding concentrations (Figure 2.6).

2.3.6 Respirometric assays

The respirometric assays are activity tests that are performed in order to measure the heterotrophic capability of the biomass for organic matter oxidation (López-Fiuza et al., 2002; Mosquera-Corral et al., 2005). The maximum specific activity (MSA) of organic matter oxidation of the biomass is obtained with these experiments.

Determination procedure

The respirometric assays are performed in a biological oxygen monitoring device (BOM5300, YSI Inc., USA) equipped with two oxygen selective probes (YSI 5331, YSI Inc., USA) (Figure 2.7).

Figure 2.7. Respirometric system: left, thermostated test cells; centre, biological oxygen monitor; right, data acquisition system.

Experiments are done at 30 °C in hermetically closed vials of 10 mL with concentrations of biomass of approximately 1 g VSS/L. The biomass is washed in a buffer solution of pH 7.0 prior to the assays to remove the possible remaining amounts of substrate of the liquid suspension media. The composition of the buffer solution is a mixture of phosphate compounds: KH_2PO_4 and K_2HPO_4 at concentrations of 0.143 and 0.74 g/L, respectively. The liquid media inside the vials is bubbled with air for at least 15 min to achieve oxygen saturation conditions (about 7.6 mg O_2/L at 30 °C). In the present case, the substrate is the mixture of pure VFAs (49:28:8:15 COD%) used for the enrichment of the heterotrophic PHA-producing biomass. The initial COD of the experiment is of 1 g COD/g VSS.

The experiment begins when the aeration is removed and the vials are tightly closed with the oxygen probes connected to a data acquisition system. It is important to check that no bubbles are present inside the vessels. Initially, the decrease of the DO concentration is registered for about 5 minutes to observe the endogenous respiration of the biomass (g O_2/(L d)). After this period, the substrate is injected (100 µL) to the vials and the oxygen consumption -due to complete biomass activity (g O_2/(L d)) is determined (López-Fiuza et al., 2002). An example of these two different slopes can be seen on Figure 2.8.

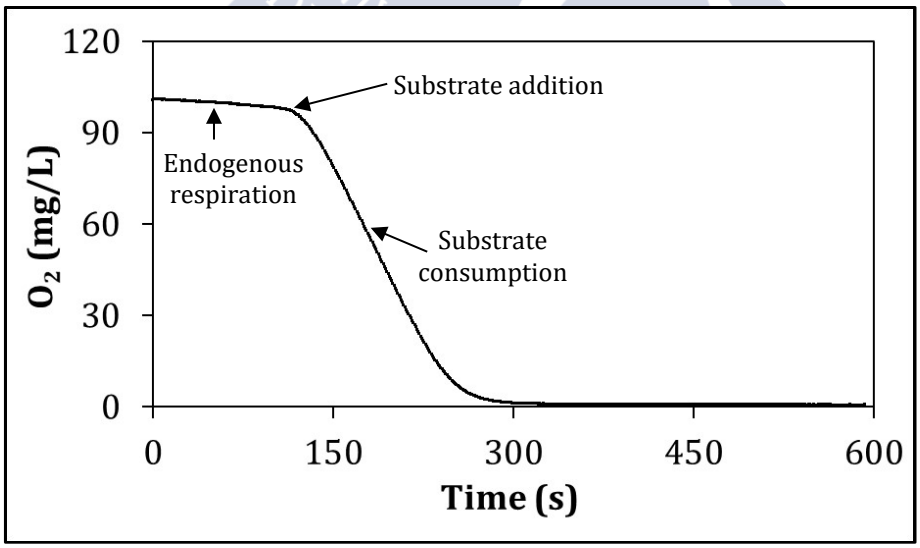

Figure 2.8. Oxygen depletion during endogenous respiration and substrate consumption.

Calculations

After the experiment, the VSS in each vial is determined according to *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). Afterwards, the specific activity of the biomass is determined by dividing the oxygen consumption rate by the VSS content (Equation 2.10).

The inhibitory effect of NaCl is measured by performing respirometric experiments with the addition of different NaCl concentrations. The IC_{50} value was estimated as the concentration of sodium which provokes an inhibition of 50% of the MSA of the biomass. The percentages of inhibition are calculated according to Equation 2.13:

$$\text{Inhibition } (\%) = \left(1 - \frac{\text{SA}}{\text{MSA}}\right) \cdot 100 \qquad \text{Equation 2.13}$$

where:

SA is the specific heterotrophic oxidation activity for each concentration tested (g O_2/(g VSS d))

MSA is the maximum specific activity corresponding to the experiment without the addition of sodium chloride (g O_2/(g VSS d))

2.3.7. Specific Methanogenic Activity (SMA)

Batch experiments were performed to measure the Specific Methanogenic Activity (SMA) of the biomass. The SMA was estimated by the online measurement of the methane (CH_4) production throughout the time of the experiment with respect to the amount of biomass present in the flasks. This method was adapted from Soto *et al*. (Soto et al., 1993).

Reagents

- Micronutrients Solution: as indicated in Table 2.4
- Macronutrients Solution: as indicated in Table 2.5

Table 2.4. Micronutrients Solution

Compound	Stock Solution Concentration (g/L)	Concentration in the flask (g/L)
$FeCl_2 \cdot 4\ H_2O$	200	$2 \cdot 10^{-3}$
$CoCl_2 \cdot 6\ H_2O$	240	$2.4 \cdot 10^{-3}$
$MnCl_2 \cdot 4\ H_2O$	60	$6 \cdot 10^{-4}$
$CuCl_2 \cdot 2\ H_2O$	5	$5 \cdot 10^{-5}$
H_3BO_3	6	$6 \cdot 10^{-5}$
$Na_2MoO_4 \cdot 2\ H_2O$	7	$7 \cdot 10^{-5}$
Na_2SeO_3	7	$7 \cdot 10^{-5}$
$NiCl_2 \cdot 6\ H_2O$	10	10^{-4}
$ZnCl_2$	5.5	$55 \cdot 10^{-6}$

Table 2.5. Macronutrients Solution

Compound	Stock Solution Concentration (g/L)	Concentration in the flask (g/L)
NH_4Cl	11.4	0.570
KH_2PO_4	8.3	0.415
$CaCl_2$	0.16	0.008
$MgCl_2 \cdot 6\ H_2O$	1.9	0.095

- Resazurin Solution 0.1 wt%
- Potassium dichromate solution 0.05 N
- Solution of 4 g/L of $Na_2S \cdot 9\ H_2O$ (it is very important to remark that this solution must be freshly prepared)
- Acetic Acid Solution at a concentration of 140 (g COD/L)
- Ethanol Solution at a concentration of 140 (g COD/L)
- Commercial Sodium Hydroxide (NaOH)

Determination procedure

Firstly, the culture media is prepared by mixing 50 mL of macronutrients solution, 10 mL of micronutrients solution, 1 mL of resazurin solution, and filling with distilled water to a final volume of 1 L. Then, each biomass sample is added to the flask (experiments done in triplicate). The bottles are sealed with aluminium caps provided with a septum. The head-space of each flask is gassed with N_2 to displace the oxygen. Afterwards, all the flasks are located in a shaker, at a temperature of 37 °C and stirred at 100 rpm. Next step is to depressurize the flasks by quickly stabbing a needle in the septum. Finally, the substrate is added to an initial biomass to substrate ratio of approximately 1 g COD/g SSV, except in the flasks corresponding to the blank experiments (prepared by triplicate). All the flasks are filled with culture media to reach the desired volume. The produced CH_4 is determined by measuring the over-pressure of the head-space with a pressure transducer (Mano 2000 Leo2, Keller-Druck, Switzerland) throughout the time. Gas samples are collected from the head-space of the and analysed by gas chromatography. Then, the amount of CH_4 produced at each time of the experiment is calculated according to Equation 2.14:

$$\mathrm{mmol}_{CH4} = \frac{P \cdot \frac{\%_{CH4}}{100} \cdot V}{0.082 \cdot (T + 273)} \qquad \text{Equation 2.14}$$

where:

P: is the pressure in the head-space indicated by the pressure transducer (atm)

$\%_{CH4}$: is the percentage of CH_4 in the biogas, measured by gas chromatography

V: is the volume of the head-space (L)

T: is the temperature of the flasks (°C)

The SMA is estimated from the maximum slope of the curve representing the amount of methane produced throughout the time (mol CH_4/h) and referred to the biomass amount present in the flask according to Equation 2.15:

$$SMA = \frac{F_{CH4} \cdot 24 \cdot 64}{VSS} \qquad \text{Equation 2.15}$$

where:

SMA: is the Methanogenic specific activity (g COD_{CH4}/(g SSV d))

F_{CH4}: is the CH_4 flow production (mmol CH_4/h)

VSS: biomass amount in the flask (g VSS)

2.4 MICROBIOLOGICAL DETERMINATIONS

The *Fluorescent In Situ Hybridization* (FISH) technique makes the identification of microorganisms at any desired taxonomical level possible, just depending on the specificity of the used probe. Nowadays, it is the semi-quantitative molecular technique most commonly used to follow microbial populations in biomass samples. The quantification of the populations is based on the use of the software called DAIME (Digital Image Analysis in Microbial Ecology) which measures total biovolume fractions of the specifically characterised populations (Daims et al., 2006).

2.4.1 Identification of bacterial populations

The FISH technique detects specific regions in the 23S or 16S rRNA with fluorescent labelled probes. These probes hybridise with the targeted sequence of any desired taxonomical level (domain, phylum, genus or specie) so they can be later detected microscopically.

A typical FISH protocol includes four steps (Amann et al., 1995): (1) the fixation and permeabilisation of the sample; (2) the hybridisation of the targeted sequence to the probe; (3) the washing steps to remove the unbound probe; and finally, (4) the detection of labelled cells by microscopy (Figure 2.9).

Reagents

- PBS (3x): is prepared by solving 0.49 g KH_2PO_4 in 80 mL of distilled water. After this, 2.3 g of NaCl are added and the pH value is adjusted to 7.2. Finally, the volume is adjusted to 100 mL. PBS (1x) is prepared by a dilution 1:3 of PBS (3x) in distilled water.
- Fixative solution: 6.5 mL of distilled water are heated to 60 °C and 0.4 g of paraformaldehyde are added. Then, one drop of NaOH 1 M is supplemented while the solution is vigorously shaken for 1-2 minutes until its complete

solubilisation. Afterwards, 3.3 mL of PBS (3x) are added and the pH value is adjusted to 7.2 with HCl (about one drop HCl 1 M). Finally, the solution is filtered through a 0.2 µm membrane filter.

- Hybridization buffer: is prepared in a 2 mL eppendorf tube by mixing: 360 µL of 5 M NaCl solution and 40 µL of 1 M Tris/HCl (pH 8.0). The amount of formamide (vol%) for the hybridisation buffer is selected depending on the probe used (Table 2.6.). Finally, 4 µL of sodiumdodecylsulfate 10% (wt/vol) are added to the mixture.
- The washing buffer: is prepared in a 50 mL Falcon tube by mixing: 1 mL of Tris/HCl (pH 8.0) and required amounts of 5 M NaCl and 0.5 M EDTA (pH 8.0) according to the percentage of formamide used with the applied probe (Table 2.7). The Falcon tube is filled up to 50 mL with distilled water and the washing buffer is preheated at 48 °C prior to its use.

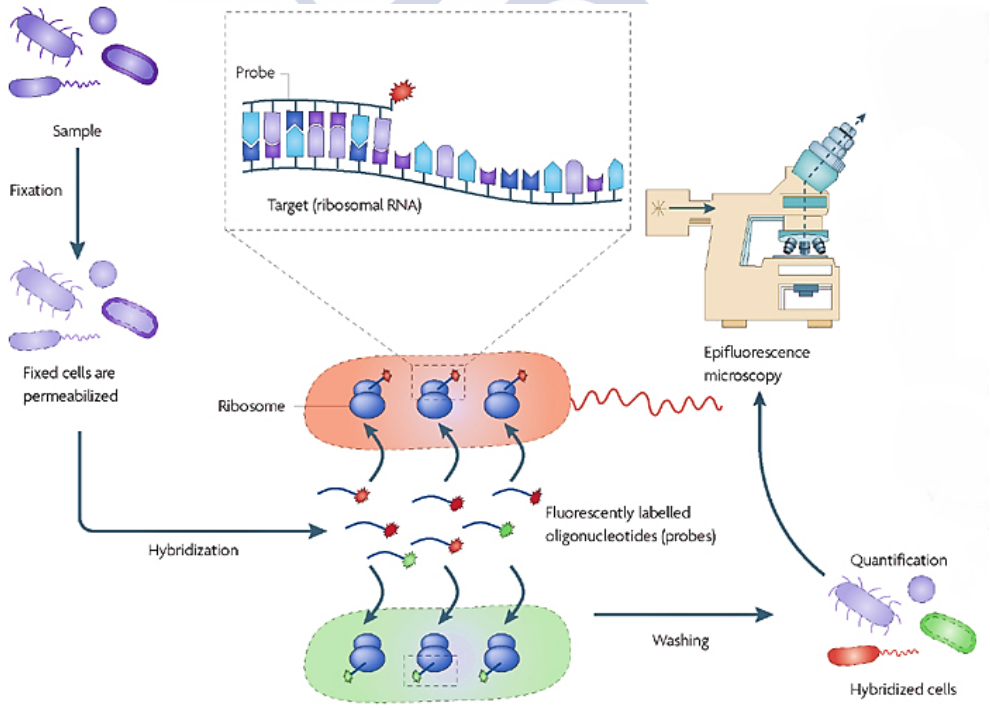

Figure 2.9. Basic steps of the FISH technique (Amann and Fuchs, 2008).

Table 2.6. Formamide and water added to the hybridisation buffer.

Formamide (vol%)	Formamide (µL)	MilliQ (µL)
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
55	1100	500
60	1200	400

Table 2.7. NaCl and EDTA added to the washing buffer.

Formamide (vol%)	5 M NaCl (µL)	0.5 M EDTA (µL)
0	9000	-
5	6300	-
10	4500	-
15	3180	-
20	2250	500
25	1590	500
30	1120	500
35	800	500
40	560	500
45	400	500
50	280	500
55	200	500
60	80	500

Procedure

1- Fixation: Fixation of the cells is essential in order to maintain the morphological integrity of the cells because the cells are exposed to high temperatures, detergents and osmotic gradients during hybridisation.
Biomass is washed in 1x PBS, then three volumes of fixative solution are added to one volume of suspension. The solution is kept on ice for 2 h. After this time, it is washed again with 1x PBS and the cells are re-suspended in 1x PBS. Iced 98% ethanol is added to the biomass suspension in a ratio 1.25:1. Samples are stored at -20 °C.

2- Immobilisation: After fixation, the cells are immobilized on a microscopic slide and used for hybridisation with 16S rDNA probes. The suspension in ethanol of the fixed biomass is spread in each well of a coated Teflon/glass microscope slide (5-10 µL). The slide is dried at 46 °C for 10 minutes. After this period, the cells are dehydrated by successively rinsing the slides 3 times for 3 minutes with 50%, 80% and 98% ethanol and dried with air.

3- Hybridisation: In order to avoid non-specific binding of the rDNA probes, the hybridization is done at stringent conditions (46 °C, 0 - 65 % formamide). The hybridisation buffer is prepared and kept at room temperature. The hybridisation tube is prepared by placing a folded tissue inside a 50 mL Falcon tube. Part of the hybridisation buffer (10 µL) is pipetted into the wells of the slides with the biomass and the rest is poured onto the tissue inside the Falcon tube. The FISH probe is added to the wells of the slides. Then each slide is placed inside the hybridisation tube and incubated for 1.5 h at 46 °C. In the meantime, the washing buffer is prepared and preheated in a water-bath at 48 °C. A tree summing up the probes available in the laboratory is shown in Figure 2.11.

4- Washing: This step should be performed rapidly: The slide is transferred into the Falcon tube containing the washing buffer and incubated for 15 minutes at 48 °C. Then the slide is removed from the washing buffer and dipped into cold distilled water for few seconds and dried with air.

5- Microscopy and image acquisition: The targeted organisms can be detected by the characteristic fluorescence of the dye (fluorochrome) attached to the probe. The fluorochromes used to detect the hybridized rRNA are FLUOS (5(6) - carboxyfluorescein - Nhydroxysuccinimide ester) and Cy3 (indo-carbocyanine). The stain DAPI (4,6-diamidino-2-phenylindole) is used for

the visualisation of all the cells present in a sample (Figure 2.10). Slide wells are embedded with Vectashield H-1200 (which amplifies the fluorescence, avoids fading and contains DAPI dye) and the cover slip is placed on the slide. For the analysis of the slides, an epifluorescence microscope (Axioskop 2 plus, Carl-Zeiss, USA) in combination with a digital camera (Coolsnap, Roper Scientific Photometrics, USA) are used. An acquisition software RSI image v 1.7.3 (Roper Scientific Photometrics, USA) is used to collect and process the images taken from the analysed samples.

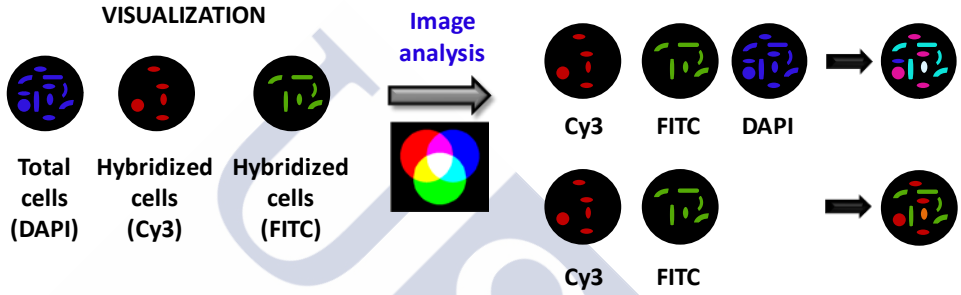

Figure 2.10. Schematic representation of the colours resulting after merging the different images corresponding to different fluorochromes.

For the characterisation of the accumulating microorganisms, the hybridisation step was performed with the following probes: EUB338mix for Bacteria, AZO644 for *Azoarcus*, CTE for *Comamonas*, MZ1 for *Thauera*, GAM42a for *Gammaproteobacteria* and PAR1244 for *Paracoccus*.

2.4.2 Quantification of bacterial populations

The quantification of the bacterial populations is based on the use of DAIME (Digital Image Analysis in Microbial Ecology) software by measuring the relative abundances (fractions of the total biovolume) of probe labelled populations in digital images (Daims et al., 2006). Although the DAIME software recommends the use of images acquired by using a confocal microscope, in this thesis, this specific software is used with images acquired by using an epifluorescence microscope, with the objective of having an approximate idea of the percentages of certain populations. The quantification is performed by comparing the positive area obtained with a specific probe with the area corresponding to the control made with DAPI probe.

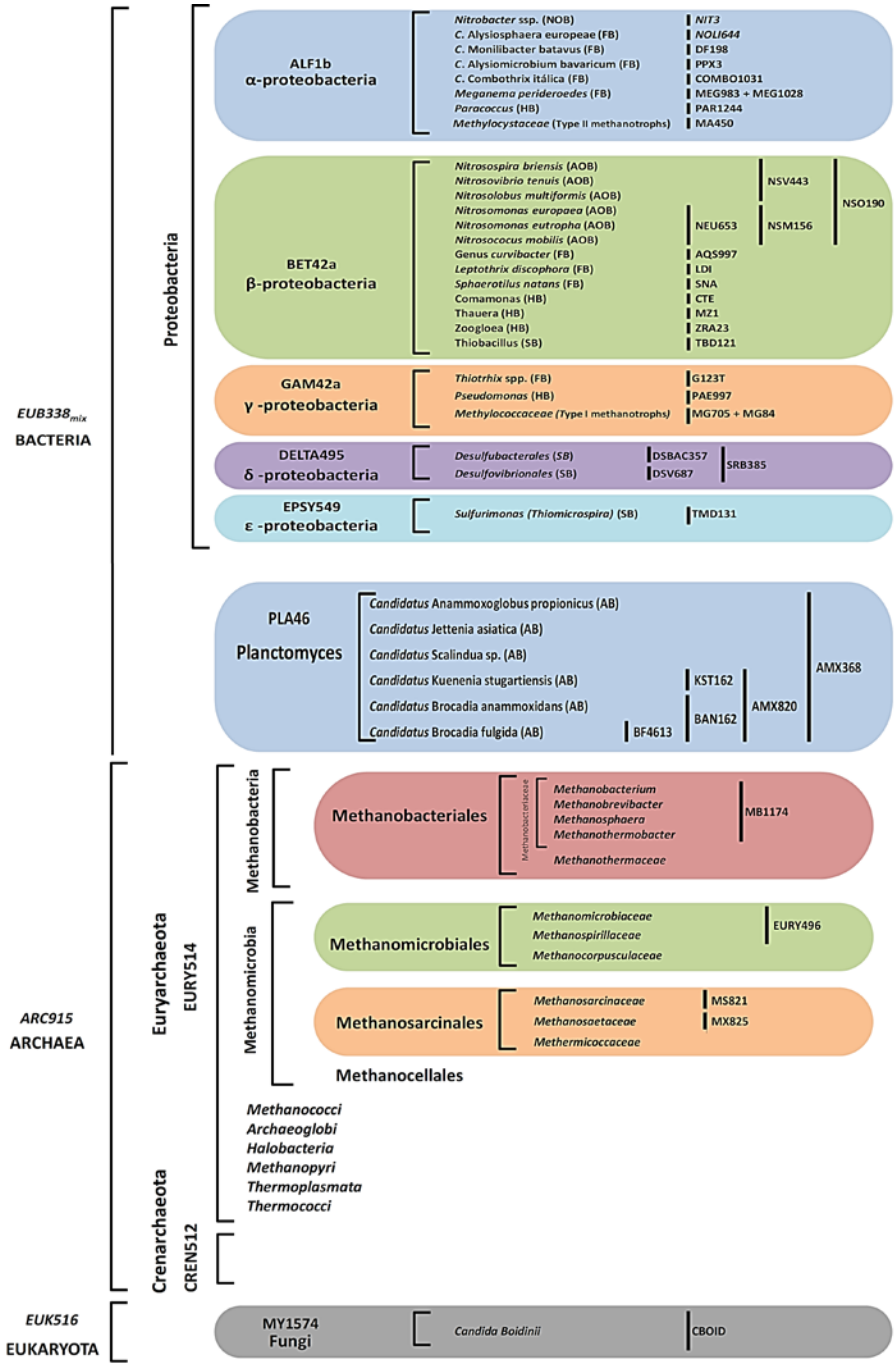

Figure 2.11. FISH probes and the main bacteria detected by them.

2.5 ENRICHMENT AND ACCUMULATION EXPERIMENTS

2.5.1 Characterisation of the Enrichment Cycles

The operational cycles of the enrichment reactors are characterised by monitoring the values of different parameters throughout single cycles. This characterisation consists of monitoring the dissolved oxygen concentration, pH, temperature, TSS and VSS, NH_4^+, the C-source concentration and the PHA production. An example of the monitoring of the enrichment cycle can be seen in Figure 2.12.

To know the values of PHA, VSS, TSS and NH_4^+ concentrations, several samples must be collected throughout the cycle. A sample before the beginning of the cycle and another once the cycle has begun (time 0) are taken. After this two first samples, several samples are periodically taken throughout the cycle. The timing for the sampling is not fixed although samples should be frequently taken during the feast phase and less often during the famine phase. This is because of the fast changes in the system during the feast stage. The concentrations of the different measured variables are determined using the methods indicated in the corresponding sections of the present chapter.

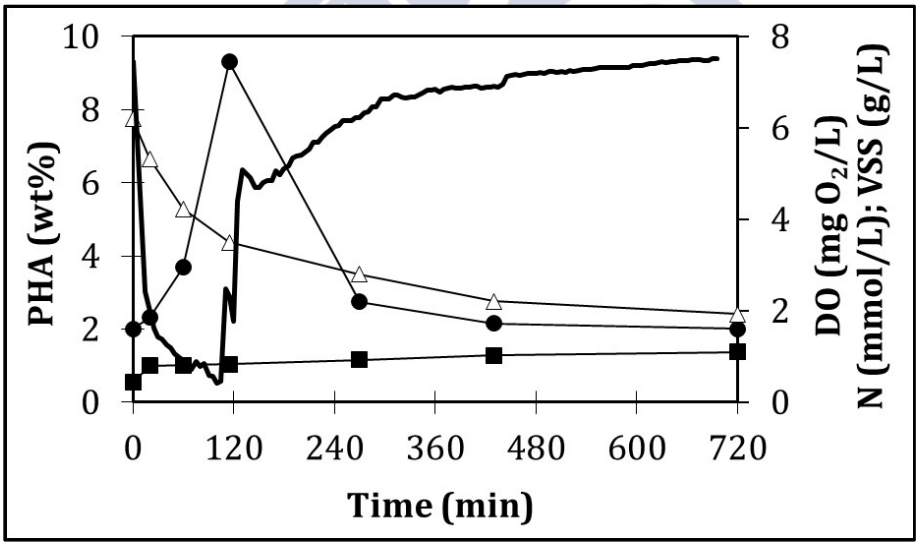

Figure 2.12. Typical representation of the different parameters measured int an enrichment cycle: NH_4^+-N concentration (Nmmol/L) (△); VSS concentration (g/L) (■); DO (-) g O_2/L) and acummulated PHA (wt%) (●).

2.5.2 Characterisation of the Accumulation Assays

For the PHA accumulation assays, the biomass is harvested from the effluent of the enrichment reactor. The quantity of the carbon source is also changed in comparison with the enrichment reactor to promote the maximum accumulation of biopolymer inside the cells. The VFAs are added in pulses every time the oxygen starts to rise after its depletion phase in the case of the fed-batch assays while they are added in one single pulse in the batch experiments. The length of the experiments is variable, depending on the biomass/substrate concentrations.

To know the values of PHA, VSS, TSS and NH_4^+ concentrations, several samples must be collected throughout the assay. Samples are taken periodically throughout the cycle. The concentrations of the different measured variables are determined using the methods indicated in the corresponding sections of the present chapter.

2.5.3 Recovery and Purification of Biopolymers

Formaldehyde was added to each biomass sample. Afterwards, samples were frozen at -20 °C and subsequently freeze-dried in a lyophilizer (Labconco, USA).

- *Recovery of PHA-films:* For the PHA-film recovery all the freeze-dried samples were suspended in chloroform. This solution was kept in an opaque vessel and maintained at 37 °C for three days in a shaker at 150 rpm. After this period, the solution was filtered and the filtrate placed in glass dishes and located in a fume hood. The evaporation of the chloroform allowed the recovery of the biopolymer as a film for further characterization. The entire process can be seen in Figure 2.13.
- *Biopolymer Purification:* A known amount of freeze-dried biomass sample was put in a tube together with 10 mL of chloroform for 2 days at 37 °C and 150 rpm, followed by a precipitation with iced ethanol in a distribution of 1:10 of chloroform:ethanol. The purification method consisted in dissolving a known amount of freeze-dried biomass sample in chloroform, followed by a precipitation with iced ethanol (Koller et al., 2013). The dissolved sample was filtered using a glass syringe coupled to a metallic filter holder with a fibreglass filter and poured in a glass beaker. Iced ethanol was added in order to precipitate the biopolymers in the form of a powder that was separated from the liquid phase by vacuum filtration. The filter with the

extracted powder was put in a hood to dry for further characterization (Figure 2.14).

Sample suspended in chloroform

Metal and glass material for filtration

Film of biopolymer

Figure 2.13. Stages of the recovery of biopolymers as films.

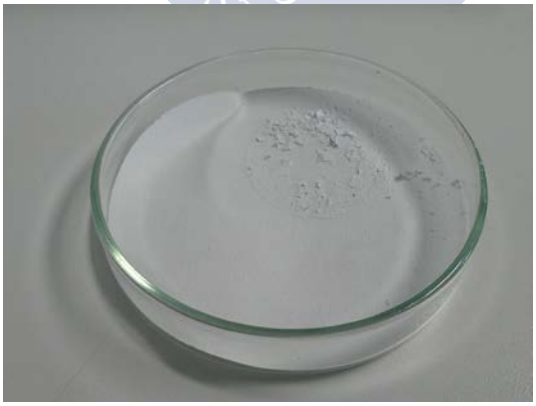

Figure 2.14. Biopolymer recovered as a powder.

- *Biopolymer impurities characterization:* The film extracted with chloroform was digested again using the modified acidic methanol method (Serafim et al., 2004) and analysed by mass spectrometry (GC-MS). Afterwards, the same film sample was purified using the chloroform-ethanol method (Koller et al., 2013; Ramsay et al., 1994). The purified biopolymer was recovered as powder and it was digested by the modified acidic methanol method so as to prepare the sample for GC-MS analysis. Chromatograms obtained by GC-MS for the samples A1 and A2, before and after the purification stage, were compared. The methylesters were analysed with a GC-MS 6850 GC Network System (Agilent Technologies, Germany) with an Agilent 5975C VL MS detector (Agilent Technologies, Germany) equipped with a DB-5 silica capillary column of 30 m x 0.25 mm (internal diameter) (Agilent-J&W Scientific, Germany).

Volatile (VSS) and total (TSS) suspended solids were analysed according to the standard methods(APHA-AWWA-WPCF, 2005). Total solids (TS) were measured by difference of weight before and after the freeze-drying process.

2.5.4 Physico-chemical properties of the biopolymers

The molecular weight distribution parameters (average molecular weight, M_w, number average molecular weight, M_n, and polydispersity, M_w/M_n) were determined by size exclusion chromatography (SEC) (Waters, USA). Chloroform was used as eluent and the system was operated at 30 °C. The equipment included a Model 6000A solvent delivery system and a mode 401 refractive index detector. A series of one pre-column and three Waters Ultrastyragel columns (10^3 Å, 10^4 Å and 10^6 Å) were used. It was performed universal calibration and used a reference substance (butylhydroxytoluene, BHT, in all the polymer samples analysed). The calibration curve was generated with nine monodisperse polystyrene (PS) standards (in the range 2 x 10^3 to 4 x 10^6, purchased from Waters and Polymer Laboratories). The calibration curve was transformed using the Mark-Houwink relationship $[\eta] = K \cdot M^a$, where $[\eta]$ is the viscosity number limit and "K" and "a" are the Mark-Houwink constants, with validity for each system polymer/solvent/temperature. The values of these constants used for the pairs PHB/chloroform and PS/chloroform, were respectively K=0.0118 mL/g; a=0.78 and K=0.0049 mL/g; a=0.794 (Kurata and Tsunashima, 2003). Polymer samples were prepared from films or powders, filtered with syringe Teflon membrane filters 0.45 μm pore size (Gelman Sciences Corp.,

USA) and injected twice; concentrations of 0.2% (wt/vol) and injection volumes of 150 μL were used.

Differential scanning calorimetry (DSC) was used in order determine the thermal properties of the obtained biopolymers in terms of T_g, T_m, and melting enthalpy (ΔH_m).The equipment used was a DSC Q2000 (TA Instruments, USA) under a nitrogen atmosphere. The samples were located in perforated aluminium pans and submitted to different heating and cooling cycles with temperatures ranging from -100 °C to 200 °C at 10 °C/min. The T_m and ΔH_m were estimated in the first heating ramp while T_g was determined in the second heating scan. In another scan the experiment was continued, increasing the temperature up to the observation of the biopolymers' decomposition. The value of the onset thermal degradation peak (endothermic) was considered the T_d.

2.6 CALCULATIONS

Calculations related to kinetic and stoichiometric values for PHA production and carbon balances are presented in this section. The amount of PHA inside the cells, biomass and CO_2, rates and yields for each produced compound are determined in each enrichment and/or accumulation batch assay. For these calculations, the units are expressed as moles of carbon by using the molecular weights of the different carbon compounds (Table 2.8). Calculations for estimating the free ammonia, sulphide, biomethane potential, and biodegradability are also presented.

Table 2.8. Molecular weights of the main C-compounds

Compound	Molecular weight (g/mol)	Molecular weight (g/Cmol)
HAc	60	30.0
HPr	74	24.7
HBu	88	22.0
HVa	102	20.4
HB	86	21.5
HV	100	20.0
Biomass*	25.1	25.1

* Biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$ (Beun et al., 2002).

2.6.1 Free Ammonia (FA)

FA concentration was calculated according to Omil et al. (1995) from the ammonium concentration and the pH value in the digester at the temperature of operation as follows (Equation 2.16):

$$FA = \frac{[NH_4^+]}{1 + \left(\frac{k_b \cdot 10^{-pH}}{k_w}\right)}$$ Equation 2.16

Anthonisen et al. (1976) stated that k_b is the ionization constant of the ammonia equilibrium equation and k_w is the dissociation constant for water, which are temperature dependant (Equation 2.17) (Anthonisen et al., 1976):

$$k_b{:}\,k_w = e^{\left(\frac{6.344}{273+T\,(°C)}\right)}$$ Equation 2.17

where the values of these two constants (k_b and k_w) at 37 °C are $1.855 \cdot 10^{-5}$ and $2.355 \cdot 10^{-14}$ respectively (Omil et al., 1995).

2.6.2 Free sulphide

Free Sulphide (FS) in the liquid phase was calculated according to Henry's Law. FS from the concentration of H_2S present in the gas phase in equilibrium with its concentration in the liquid phase (Equation 2.18):

$$P_i = k_{Hi} \cdot C_i$$ Equation 2.18

where:

P_i: is the H_2S partial pressure (atm H_2S).

C_i: is the H_2S concentration in the liquid phase (g H_2S/L).

k_{Hi}: is the Henry's constant and it has a value of 0.375 atm H_2S/(g H_2S · L) at 37 °C (Omil et al., 1995).

The sulphide in the liquid phase is allocated in S^{2-}, HS^- and H_2S. All these three species make up for the Dissolved Sulphide (DS). The equilibrium reactions are detailed as follows (Equation 2.19; Equation 2.20) (Omil et al., 1995):

$$H_2S \overset{k_1}{\leftrightarrow} HS^- + H^+$$ Equation 2.19

$$HS^- \overset{k_2}{\leftrightarrow} S^{2-} + H^+$$ Equation 2.20

where:

k_1 is the equilibrium constant for (Equation 2.19) and it is calculated with the following equation (Equation 2.21):

$$k_1 = [(0.382 \cdot T(°C) + 1.892) \cdot 10^{-8}] \frac{mol}{L}$$ Equation 2.21

k_2 is the equilibrium constant for (Equation 2.20) and it is calculated with the following equation (Equation 2.22):

$$k_2 = [1 \cdot 10^{-19}] \frac{mol}{L} \text{ (at 25 °C)}$$ Equation 2.22

In a range of pH values between 7 and 8, only HS^- and H_2S are considered since the amount of S^{2-} present under these conditions is negligible. Dissolved Sulphide (DS) was calculated according to Isa et al. (1986) from the FS concentration considering Equation 2.19:

$$k_1 = \frac{[H^+][HS^-]}{[H_2S]}$$ Equation 2.23

$$pH = -\log[H^+] \rightarrow [H^+] = 10^{-pH}$$ Equation 2.24

where k_1 is the equilibrium constant from (Equation 2.19) and it has a value of $1.49 \cdot 10^{-7}$ at 35 °C (Isa et al., 1986).

The relationship between the FS concentration ($[HS^-]$) and the DS concentration ($[H_2S]+[HS^-]$) is calculated from using the above k_1 (Equation 2.23) and the pH equation (Equation 2.24). The k_1 value is the indicated in Equation 2.23 for 35 °C or using Equation 2.21 for any change of the temperature. Doing the calculations, the following equation is obtained (Isa et al., 1986):

$$\frac{FS}{DS} = \frac{1}{\left(1 + \frac{k_1}{10^{-pH}}\right)}$$ Equation 2.25

2.6.3 BioMethane Potential and Biodegradability

The biomethane potential (BMP) and the biodegradability (BD) of the substrate were determined for the continuous operation of the anaerobic reactor.

The BMP is determined as the volume of methane produced per mass of substrate fed as COD (L CH_4/kg COD_{fed}).

The biodegradability (BD) of the substrate was determined by dividing the real production of CH_4 by its theoretical production (Mottet et al., 2010). The estimated theoretical value used is 350 L CH_4/kg COD_{fed} considering that the recovered methane represents between 85- 95% of the COD removed (Lema et al., 1992).

2.6.4 Carbon balances

Mass balances are applied to the results obtained from the parameters monitored during cycle measurements, which are calculated for enrichment and accumulation reactors. The consumed carbon, related to VFAs depletion, should be the same carbon associated to PHA production, CO_2 generation and biomass growth.

PHA calculations

The content of each monomer (HB or HV) accumulated inside the biomass samples is expressed as a percentage of the measured amount of compound related to the solids amount in the sample (wt%) and calculated in Equation 2.11. A detailed description of the proceedings and calculations are indicated in Section 2.3.2.

Biomass

Ammonium consumption is fully attributed to biomass growth due to the addition of ATU which inhibited the nitrification process. In this way, the amount of produced biomass is proportionally related to the ammonium consumption, assuming a biomass composition adjusted to the stoichiometry of $CH_{1.8}O_{0.5}N_{0.2}$ (Beun et al., 2002). This means that every N-mol consumed generates 5 C-mol of biomass. The active biomass is expressed as X in the following equations and calculations.

CO_2 calculations

The amount of CO_2 generated during the process is estimated stoichiometrically. The CO_2 generated during the feast phase is calculated in the same way as the CO_2

generation in the accumulation experiments. It is assumed to be the CO_2 produced by the biomass growth plus the CO_2 generated due to PHA production.

$$0.9080\ \left(\frac{\text{Cmol } CO_2}{\text{Cmol X}}\right) \cdot \frac{5}{1}\left(\frac{\text{Cmol X}}{\text{Nmol}}\right) \cdot \Delta\text{N (Nmol)} + 0.35\ \left(\frac{\text{Cmol } CO_2}{\text{Cmol PHA}}\right) \cdot \text{Cmol PHA} \qquad \text{Equation 2.26}$$

The CO_2 generated during the famine phase is calculated as the CO_2 produced by the biomass growth plus the CO_2 generated due to PHA consumption:

$$0.4965\ \left(\frac{\text{Cmol } CO_2}{\text{Cmol X}}\right) \cdot \frac{5}{1}\left(\frac{\text{Cmol X}}{\text{Nmol}}\right) \cdot \Delta\text{N (Nmol)} + (-1)\ \left(\frac{\text{Cmol } CO_2}{\text{Cmol PHA}}\right) \cdot \Delta\text{PHA (Cmol PHA)} \qquad \text{Equation 2.27}$$

2.6.5 F/M ratio, rates and yields

Rates and yields are calculated from the experimental assays obtained from enrichments and accumulations. The F/M ratio was calculated in terms of (Cmol VFA/Cmol X).

Rates and Specific Rates

VFA consumption rates (Cmol VFA/h) are calculated from the slopes of the curves representing the experimental data obtained from each cycle measurement assay and describing the intake of VFAs throughout time (Figure 2.15). The values of these rates are divided by the biomass content in the experiment in order to obtain the specific substrate uptake rates (Cmol VFA/(Cmol X · h)).

A similar procedure is applied for calculating the active biomass (X) specific growth (Cmol X_{new} /(Cmol X · h)); the CO_2 specific production rate (Cmol CO_2/(Cmol X · h)); and the HB and HV specific production rates (Cmol HA/(Cmol X · h)). Yields for HB and HV (Cmol HA/Cmol VFA) are obtained from the corresponding experimental data by dividing the polymer production rate (Cmol PHA/h) by the VFA consumption rate (Cmol VFA/h). A similar modus operandi is followed to estimate the produced CO_2 (Cmol CO_2/Cmol VFA) and the biomass growth (Cmol X_{new}/Cmol

VFA). The average active biomass concentration (X) is estimated as the difference between the VSS and the PHA content in the cells.

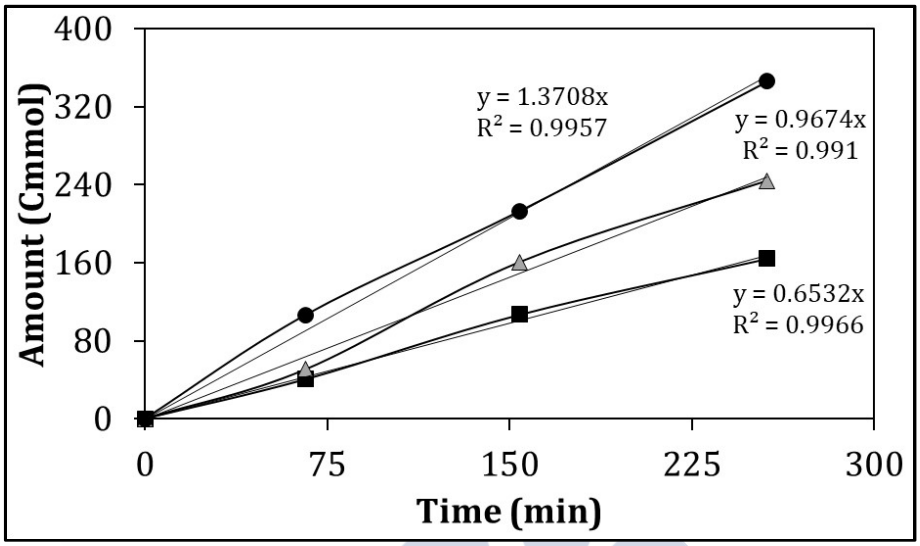

Figure 2.15. Experimental data obtained from an accumulation assay with the equations of the represented data: HV (Cmmol) (■); HB (Cmmol) (△); and substrate consumed (Cmmol) (●).

Yields

To calculate the HB and HV yields, a representation like Figure 2.15 is needed. Then, the slopes of PHA production are divided by the slope of the VFA consumption and the value of the corresponding yields is obtained in units of Cmol HB/Cmol VFA and Cmol HV/Cmol VFA, for hydroxybutyrate and hydroxyvalerate, respectively. The same procedure is applied for the calculation of the X and CO_2 yields, with their results in Cmol X/Cmol VFA and Cmol CO_2/Cmol VFA, respectively.

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Chapter 3
METHANE PRODUCTION UNDER SALINE CONDITIONS

SUMMARY

The presence of salts in wastewaters is common in coastal areas, where leakages of salty water occur into the sewage systems, or in certain industrial sectors like the fish canning industry. Aerobic granular sludge (AGS) systems can be suitable to treat brackish wastewaters as an alternative to activated sludge. These systems accumulate large biomass concentrations which enable the counteraction of the decrease of the activity due to salt presence. These AGS systems have also as an advantage the lower amount of sludge generated in comparison with the activated sludge ones. Although the excess of sludge produced needs to be post-treated. The anaerobic digestion (AD) process is normally used to reduce the concentration of solids and to produce biogas. Up to date, the effects of the presence of relevant salt concentrations in AD systems has been studied in for the wastewater treatment but scarcely revised when treating sewage sludge.

The aim of this work is to evaluate the biomethane potential (BMP) and the feasibility of the anaerobic biodegradation of the aerobic granular sludge (AGS) under brackish conditions and to compare the results with those from the AD of flocculent activated sludge (FLAS). The obtained values of the biodegradability (BD) of AGS (32%) and FLAS (27%) were similar, which indicates that the aggregation state of the substrate did not limit the process. Brackish conditions led to a concentration of sodium and free sulphide (FS) inside the reactor in the range of 2.1-5.2 g/L and 38-93 mg/L, respectively. These values are within the inhibitory levels reported for the anaerobic treatment of wastewaters. However, the BMP and the BD obtained in this work indicated that neither sodium nor free sulphide had an inhibitory effect at these concentrations. The content of H_2S in the biogas was relevant (1.5-3.8%) and its pre-treatment is needed if the biogas is going to have any further use, i.e., in energy production.

Outline

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3.1 INTRODUCTION

Many industrial wastewaters contain high salt concentrations which cause negative effects on biological organic matter, nitrogen and phosphorus removal processes (Intrasungkha et al., 1999; Uygur and Kargı, 2004). The presence of large salts concentrations, due to punctual infiltrations of seawater in the sewer systems located near the coast, can provoke disturbances of the performance of urban wastewater treatment plants (WWTPs). These salinity shocks can affect the microbial populations in the sludge and also the quality of the effluent in terms of suspended solids (Hamoda and Alattar, 1995; Tokuz and Eckenfelder, 1979). The AGS systems can be a suitable technology to treat brackish wastewater since the large biomass concentrations achieved compensate by the decrease of the biomass activity found under saline conditions. In fact, these granular systems have been tested recently to remove phenol from saline wastes (Moussavi et al., 2010) and also to treat fish canning effluents (Figueroa et al., 2008).

New technologies using aerobic granular sludge have been found to also reduce the sludge production significantly in comparison with conventional activated sludge systems (Campos et al., 2009). This is an important issue because the sludge disposal represents up to 50% of the operational costs in a WWTP (Appels et al., 2008). Post-treatment of the produced biomass in excess is still necessary although the sludge production of the AGS systems is lower than with conventional processes. Several disposal and treatment methods are available. Due to the special properties of the granules, like their high hydrophobicity and good settling properties (Val del Rio et al., 2011), thickening and/or dewatering constitute feasible options. The anaerobic digestion (AD) is also an excellent option because the sludge resulting from wastewater treatment systems is rich in nutrients and organic matter. The AD is conventionally used for the treatment of organic wastes because of its simplicity with the asset of biogas production (Lettinga, 1995). It also contributes to the stabilization of the treated sludge by destroying most of the pathogens. So the AD could be a suitable option to treat the sludge generated by the aerobic granular sludge systems having in mind that the rate-limiting step is the hydrolysis of complex substances present in the solids (Mata-Alvarez et al., 2000). In this way, an earlier study (Val del Rio et al., 2011) showed that the anaerobic biodegradability of the granular sludge was similar to that reported for waste activated sludge, although these work has been carried out in batch experiments and in the absence of saline conditions.

The salinity can affect not only the biological processes performance in the water line but also the efficiency of the anaerobic digestion during sludge treatment (King, 1982). Until now, the effect of saltine conditions on the anaerobic digestion was preferentially studied in systems treating industrial wastewater. A previous research work (Feijoo et al., 1995) has reported in batch assays the decrease of the methane production in the presence of sodium concentrations higher than 2 g Na^+/L. Although, it is important to remark that to consider the aggregation state of the biomass and the acclimation phenomena are essential in order to compare results. The toxicity of the generated sulphide and the competition between sulphate reducing bacteria (SRB) and methane producing microorganisms (MPM) during wastewater treatment have been also widely reported in literature (Chen et al., 2008; Isa et al., 1986). Nevertheless, the effect of the presence of brackish water in the performance of the anaerobic digesters treating sewage sludge has been scarcely studied and most of the studies have been performed with sludge generated in aquaculture installations (Gebauer, 2004; Mirzoyan et al., 2008).

3.2 OBJECTIVES

Therefore, the aim of the present research work is to study the performance of an anaerobic digester treating two kinds of biomass under brackish conditions: aerobic granular sludge (AGS) and flocculent activated sludge (FLAS). The effect of sodium and sulphide concentrations over the biodegradability (BD) of the sludges is tested.

3.3 MATERIALS AND METHODS

3.3.1 Experimental set-up

A fed-batch anaerobic digester (Álamo, Spain) with a useful volume of 5 L was used (Figure 3.1). The reactor was inoculated with 18 g SSV/L of suspended sludge from an anaerobic digester treating the wastewater from a brewery sited in A Coruña, Spain.

It was operated in complete mixing conditions by means of a mechanical stirrer (Heidolph, Germany) functioning at 100 rpm. The operational temperature was set in the mesophilic range at 35 ºC using a thermostatic bath connected to the reactor jacket (Techne Inc., USA). The pH value was neither measured on-line or controlled. The volume of the produced biogas was measured with a flow-meter based on water

displacement measurements (Veiga et al., 1990) provided with two electrodes connected to a digital meter (Omron, Japan).

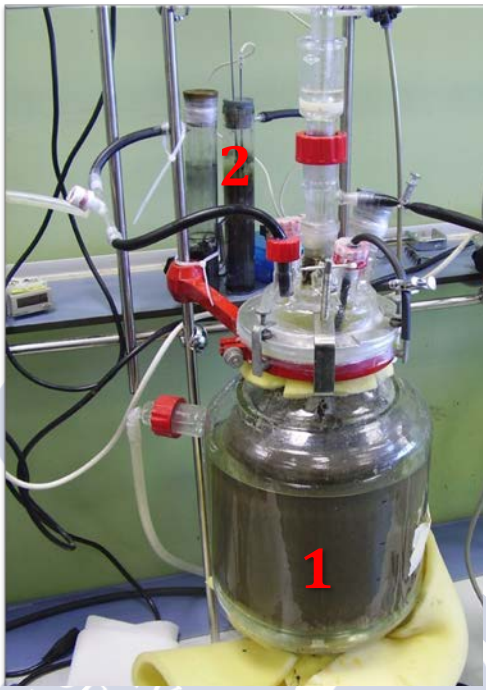

Figure 3.1. Anaerobic sludge digestor (1) system with the biogas flowmeter (2).

The reactor operated at a hydraulic retention time (HRT) of 20 days. The digester was firstly fed with AGS and afterwards with FLAS. The AGS was collected periodically from an aerobic granular pilot plant (100 L) that treated pig slurry to remove organic matter and nitrogen, which was operating in the University of Santiago de Compostela. The operational conditions of this plant are described elsewhere (Jungles et al., 2011). The FLAS was taken from the activated sludge system of an urban WWTP situated in Calo-Milladoiro (A Coruña, Spain). In order to have brackish conditions inside the reactor, the supernatant was replaced by seawater so as to obtain similar salt concentrations than those observed in a coastal urban WWTP during seawater infiltrations or discharges coming from nearby fish-canning industries. Both feedings were stored at 4 ºC in order to avoid degradation processes.

The feeding and withdrawal of the treated sludge were manually performed three times per week to avoid the clogging of the pipes. The use of peristaltic pumps was also eluded because they could disintegrate the granules when treating AGS.

3.3.2 Operational Conditions

The operation of the reactor throughout the time was divided in three major stages and their corresponding sub-stages (Table 3.1), depending on the type of sludge fed and/or the salts concentrations.

In the first stage (Stage 1), the anaerobic digester was operated for 233 days treating AGS containing increasing salt concentrations in the range from 2.1 to 3.0 g Na^+/L and from 0.5 to 0.7 g SO_4^{2-}/L, together with increasing organic loading rates (OLR) from 1.2 to 1.6 g COD/(L · d) (Sub-stages AGS1-3).

In the second stage (Stage 2), the anaerobic digester was operated for 40 days treating the same AGS but with salt concentrations higher than in the first stage in order to study its short-term influence in the performance of the anaerobic digester. The concentrations assayed of the main salts were 6.7 and 5.4 g Na^+/L with 1.5 and 1.2 g SO_4^{2-}/L for each sub-stage (AGS4 and 5), respectively. Applied OLRs were lower than in Stage 1 of 0.4 and 0.9 g COD/(L · d).

In the third stage (Stage III), the anaerobic digester was operated for 96 days treating FLAS with salt concentrations similar to those used in the first stage (2.6 g Na^+/L and 0.7 g SO_4^{2-}/L) in order to compare the anaerobic degradation of aerobic granular and flocculent sludges under saline conditions. The applied OLR was also similar of 1.5 g COD/(L · d).

Table 3.1. Characteristic parameters of the sludge fed to the anaerobic digester in the different operational periods.

	Stage I			Stage II		Stage III
	AGS1	**AGS2**	**AGS3**	**AGS4**	**AGS5**	**FLAS**
Days of operation (d)	1 - 63	64 - 163	164 - 233	234 - 250	251 - 273	274 - 369
Operational conditions						
Applied OLR (g COD/(L · d)	1.2 ± 0.1	1.3 ± 0.1	1.6 ± 0.1	0.4 ± 0.0	0.9 ± 0.0	1.5 ± 0.2
Fed sludge characteristics						
TS (g/L)	18.8 ± 3.8	21.1 ± 2.1	16.4 ± 1.7	12.1 ± 1.0	14.0 ± 1.4	15.7 ± 4.3
VS (g/L)	14.5 ± 3.1	15.9 ± 2.0	12.2 ± 0.9	9.8 ± 4.7	7.3 ± 0.9	10.9 ± 3.9
VS/TS (%)	76.9 ± 3.8	76.2 ± 4.4	76.2 ± 2.6	63.5 ± 4.8	53.0 ± 0.6	65.8 ± 1.9
COD (g/L)	23.6 ± 1.4	26.1 ± 0.5	31.1 ± 2.7	8.0 ± 0.7	18.6 ± 0.5	29.0 ± 4.3
COD:VS (g/g)	1.6 ± 0.5	1.7 ± 0.2	1.7 ± 0.2	1.3 ±0.2	1.3 ± 0.1	1.2 ± 0.6
ϕ (mm)	1.6	1.7	1.7	1.4	1.4	-
Salinity of the liquid media						
$[Na^+]$ (g/L)	2.1 ± 0.5	2.4 ± 0.6	3.0 ± 0.1	6.7 ± 1.2	5.4 ± 0.2	2.6 ± 1.0
$[SO_4^{2-}]$ (g/L)	0.5 ± 0.0	0.6 ± 0.1	0.7 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	0.7 ± 0.1
$COD:SO_4^{2-}$ (g/g)	47	43	47	5	15	45

ϕ: Average diameter of the granules contained in the AGS

3.3.3 Analytical methods

Total and Soluble Chemical Oxygen Demand (COD_T and COD_S, respectively) were determined following the method 5220C of the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005) modified for samples with high concentration of salinity (Soto et al., 1989). The concentrations of VFAs were measured following a the method described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005) by gas chromatography (GC) (Hewlett Packard 5890A, USA). The alkalinity was determined following the method described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005).

The concentrations of total ammonium (NH_4^+-N) were determined spectrophotometrically (Bower and Holm-Hansen, 1980) and those from other ions, like sodium (Na^+), potassium (K^+) and sulphate (SO_4^{2-}), by ionic chromatography (IC). The pH was measured with an electrode (52-03, Crison Instruments, USA) and the temperature with a digital thermometer.

Biogas composition was analysed by GC (Hewlett Packard 5890A, USA). The biogas production was measured continuously with a flow-meter (Veiga et al., 1990).

The concentrations of Total (TSS) and Volatile Suspended Solids (VSS) were determined according to the methods described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005).

All these methods are described in detail in Chapter 2 “Materials and Methods”.

3.3.4 Calculations

Free ammonia (FA) and free sulphide (FS) were determined according to Omil et al. (1995). The biomethane potential (BMP) and the BD were also calculated. All these calculations are described in detail in Chapter 2 “Materials and Methods”.

3.4 RESULTS AND DISCUSSION

3.4.1 Anaerobic Digester Operation

A continuous stirred and jacketed reactor of 5 L was operated for 369 days at a HRT of 20 days treating, under brackish conditions, firstly AGS and secondly FLAS.

By the time of the performance of this study, there were no previous works dealing with the anaerobic digestion of AGS in a continuous operation mode except for an earlier study which proved the feasibility of the anaerobic digestion of AGS (Val del Rio et al., 2011) but in batch assays and under non-saline conditions.

During all the operational period, the pH value and the concentrations of VFA, TA and FA (Table 3.2) were measured regularly as indicators of the digester performance. The pH ranged between 7.0 and 7.3, which is inside the optimal range of 6.8-7.4 reported for the anaerobic sludge digestion (Dohànyos and Zábranská, 2001). The VFA concentration inside the reactor was below the detection levels in Stages I and II, while in Stage III a concentration of 0.27 g COD/L as propionic acid was measured from day 331 to day 341. The TA during the operational period was around 3 g/L which falls between the favourable range of values which range from 1 to 5 g $CaCO_3$/L (Dohànyos and Zábranská, 2001). The FA concentration was always lower than the inhibitory value of 20 mg NH_3-N/L for the anaerobic digestion process (Gallert and Winter, 1997).

The anaerobic digester was fed during Stage I with AGS and salts concentrations up to 3.0 g Na^+/L and 0.7 g SO_4^{2-}/L (Table 3.1). It was started up at an OLR of 1.2 g COD/(L · d). The load was progressively increased up to a final value of 1.6 g COD/(L · d) (Table 3.1) during three sub-stages defined as AGS1, AGS2 and AGS3 which correspond to each new batch of AGS collected from the pilot plant. The average BMP during the sub-stage AGS1 was 134 ± 25 L CH_4/kg COD_{fed} but in sub-stage AGS2 the BMP value was appreciably lower (78 ± 33 L CH_4/kg COD_{fed}). A similar trend was observed in the case of the BD, which decreased from 42.3 to 23.1%. This decrease could be related to the large straw content of the pig manure treated in the aerobic granular pilot plant, which accompanied the sludge used in this second sub-stage. This straw was not quantified but it could be appreciated embedded in the surface of the granules fed to the anaerobic digester. At the end of Stage I a new batch of AGS was used containing less straw than the previous one and this was reflected in the

observed increase of the BMP to the average value of 93 ± 30 L CH_4/kg COD_{fed} (BD of 31.7%).

In Stage II, the reactor was fed with AGS containing higher salts concentration than in the previous stage at values up to 6.7 g Na^+/L and 1.5 g SO_4^{2-}/L (Table 3.1). The applied OLRs were significantly lower than in Stage I (0.4 and 0.9 g COD/(L · d)) due to the lower concentration of AGS (in terms of volatile solids) collected from the pilot plant throughout this period. The overall BMP in Stage II reached an average value of 130.5 L CH_4/kg COD_{fed} which was significantly high compared to the BMP obtained in the previous stage. However, the relevance of this results must be carefully evaluated as it might be not representative in relation to the operational conditions of Stage II considering the fact that both sub-stages (AGS4 and 5) lasted around 20 days each which is the length of the HRT of operation. For these reasons, the increase of the BMP during this Stage II could be attributed to the degradation of the non-digested COD remaining inside the reactor from the previous stage, which could provide an overestimation of this parameter even at higher concentrations of sodium and sulphide.

The SMA of the biomass degrading the AGS was estimated under non-brackish conditions as 0.016 L CH4/(g VS · d), which resulted similar to the value corresponding to the biomass adapted to saline conditions, collected during Stage II from the reactor, of 0.015 L CH_4/(g VS · d). On the one hand, this agrees with the fact that no VFAs were accumulated during the operation of the present system and, on the other hand, it proves the absence of negative effect of saline conditions on the SMA. In this way, the low methanogenic rates observed point out to the hydrolytic step as the limiting one. These SMA values are in accordance with the values that other authors have observed (0.010 – 0.020 L CH_4/(g VS · d)) when treating saline effluents from fish farms with high content in particulate organic matter (Gebauer, 2004).

In order to compare the anaerobic digestion of granular and flocculent sludge under saline conditions, the digester was fed during Stage III with FLAS at similar salt concentrations and applied OLR to those imposed in Stage I (Table 3.1). The average BMP during this last stage was 94 ± 53 L CH_4/kg $COD_{fed,}$ which is a value similar to the overall value obtained in Stage I treating the AGS (103 ± 39 L_{CH4}/kg COD_{fed}). However, some operational issues occurred only when digesting the FLAS in

continuous mode related to the formation of foam in the upper zone of the reactor which provoked the hindering of the biogas outflow and the overflowing of foam.

Regarding the fact that the degraded sludges were one granular and the other flocculent the lower surface to volume ratio of the former in comparison with the latter, seems not to affect the hydrolysis step of the anaerobic digestion in the range of sizes of the granules used in this work (Table 3.1). The obtained results show that the anaerobic BD of AGS and FLAS under similar brackish conditions were comparable (32% and 27%, respectively) which indicates that the aggregation state of the biomass did not influence the anaerobic digestion performance. The lower apparent values of BD and BMP during Stage III in comparison with the values obtained during Stage I can be explained from the point of view of the operation because the reactor treating AGS led to much lower scum formation than when treating FLAS, which avoided the operational problem of the biogas hindering.

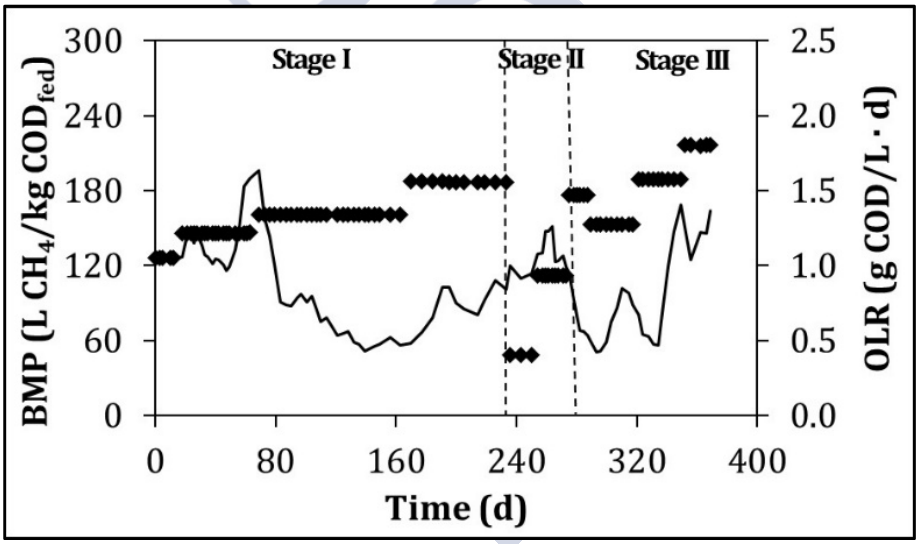

Figure 3.2. Evolution of the BMP(L CH_4/kg COD_{fed}) (-) and the ORL (g COD/(L · d)) (◆) through time.

Table 3.2. Average values of the characteristic operational parameters measured throughout the different operational stages.

	Stage I			Stage II		Stage III
	AGS1	**AGS2**	**AGS3**	**AGS4**	**AGS5**	**FLAS**
Days of operation (d)	1 - 63	64 - 163	164 - 233	234 - 250	251 - 273	274 - 369
pH	7.2 ± 0.1	7.1 ± 0.1	7.2 ± 0.2	7.1 ± 0.1	7.2 ± 0.1	7.1 ± 0.1
VFA (g COD/L)	ND	ND	ND	ND	ND	0.27*
TA (g $CaCO_3$/L)	3.1 ± 0.5	3.0 ± 0.5	2.6 ± 0.2	2.8 ± 0.1	3.4 ± 0.0	2.3 ± 0.4
FA (mg NH_3-N/L)	9.4 ± 2.3	9.8 ± 3.4	9.9 ± 2.4	8.1 ± 1.2	9.6 ± 1.7	8.45 ± 2.8
BMP (L CH_4/kg COD_{fed})	134 ± 25	78 ± 33	93 ± 30	125 ± 58	136 ± 31	94 ± 53
BD (%)	42.3 ± 10.7	23.1 ± 6.6	31.7 ± 8.9	36.8 ± 17.0	40.1 ± 9.4	26.8 ± 12.5
$Na^+_{reactor}$ (g/L)	2.3 ± 0.5	2.3 ± 0.6	2.7 ± 0.1	5.0 ± 0.7	5.2 ± 0.3	2.9 ± 0.4
Biogas composition						
CH_4 (%)	59.3 ± 2.9	55.7 ± 4.2	56.1 ± 5.3	58.0 ± 2.9	61.9 ± 3.3	61.8 ± 3.0
H_2S (%)	1.5 ± 0.8	1.5 ± 1.0	1.9 ± 0.7	3.5 ± 1.1	3.8 ± 0.4	2.1 ± 0.6
Sulphur Compounds						
[SO_4^{2-}] (g/L)	0.10 ± 0.04	0.10 ± 0.07	0.19 ± 0.13	0.15 ± 0.09	0.39 ± 0.09	0.11 ± 0.06
DS (mg H_2S-S/L)	164 ± 85	128 ± 87	185 ± 62	287 ± 128	347 ± 34	170 ± 70
FS (mg H_2S-S/L)	42.4 ± 18.3	38.2 ± 26.0	56.7 ± 18.5	91.5 ± 49.0	93.1 ± 11.0	54.1 ± 16.3
$SO_4^{2-}{}_{,removed}$(%)	80.2 ± 8.4	83.0 ± 10.8	70.9 ± 19.7	89.8 ± 5.8	67.2 ± 7.2	82.6 ± 9.3

ND: Not Detected. *Only detected as propionic acid between days 331-341 of operation.

3.4.2 Influence of the salinity in the overall operation

The AD of organic solids mixed with brackish water can be problematic due to the presence of different dissolved salts like sodium or sulphate. Sodium is indispensable for methanogenic microorganisms but high concentrations of this element are inhibitory. It is assumed that concentrations in the range of 0.1-0.4 g Na^+/L are stimulatory for the AD process, concentrations between 3.5-5.5 g Na^+/L are moderately inhibitory and over 8 g Na^+/L strongly inhibited the methanogenic microorganisms (Appels et al., 2008; Chen et al., 2008). Total inhibition of the process has been reported from 10 g Na^+/L in a mesophilic continuous reactor treating fish farming sludge (Gebauer, 2004). Although, sodium inhibition extension on AD strongly depends on its concentration several studies reveal that other agents influence its effect. These factors influencing the tolerance of anaerobic sludges to sodium are acclimation time, the aggregation state of the biomass and also the operational mode of the reaction system (Feijoo et al., 1995).

In the present study, the concentration of sodium inside the reactor throughout Stages I and III was moderate with an average value of 2.3 and 2.9 g Na^+/L, respectively. Marked inhibitory concentrations where applied in Stage II, with values in the feedings of 6.7 and 5.4 g Na^+/L in the sub-stages AGS4 and AGS5, respectively. These values did not affect significantly the performance of the anaerobic degradation of the organic matter. It is important to remark for sub-stage AGS4 that the concentration of sodium inside the reactor (5.0 g Na^+/L) was lower than in its respective feeding (6.7 g Na^+/L, Table 3.1) because the operational period when treating this sludge was shorter than the applied HRT and the maximum concentration of sodium in the reactor was not reached.

With respect to sulphate, high concentrations of this ion can affect the AD at two different levels: one occasioned by the competition for organic matter between SRB and anaerobic microorganisms, and the other one as a result of the sulphate reduction to sulphite, which is toxic especially to methanogenic microorganisms (Chen et al., 2008). The SRB are able to metabolize fatty acids, alcohols, etc. but they are not capable of degrading biopolymers. For this reason the competition does not occur in the hydrolytic stage of the anaerobic digestion but the SRB are relevant during the acidogenic and methanogenic stages because these consortia compete for the same fermentation products (Appels et al., 2008). Several factors, like the

presence of sulphide, the acclimation or the COD:Sulphate ratio, influence the extension of this competition. Some authors, treating fish canning wastewaters in a semi-continuous mode, stated that the affectation depends more on the COD:Sulphate ratio than on the absolute sulphate concentration and its conversion to sulphide (Soto et al., 1991). A common recommendation is to operate at COD:Sulphate ratios higher than 10 g COD/g SO_4^{2-} (Omil et al., 1995) because lower ratios are thought to be inhibitory for methanogenesis operated in mesophilic conditions. The ratios used in this work were always above the advised value of 10 g COD/g SO_4^{2-} (Table 3.1) in order to minimize the competition between SRB and MPM with the exception of AGS4 stage. The ratio in this sub-stage was 5 g COD/g SO_4^{2-} (Table 3.1).

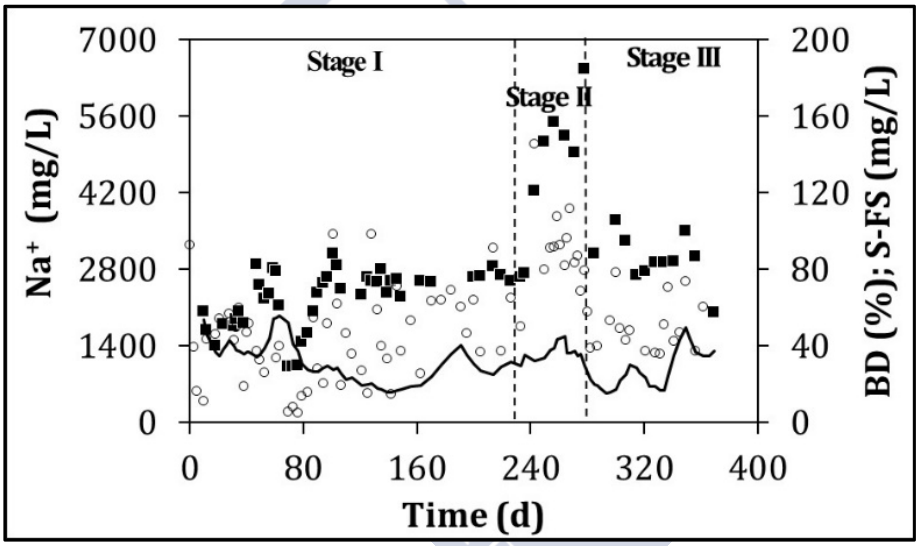

Figure 3.3. Evolution of sodium (mg/L) (■), sulphide (mg/L) (○)and BD (%) (-) through time

The reduction of sulphate to sulphide when treating sludge under brackish conditions is kinetically and thermodynamically favoured by the operation of the anaerobic digestion in mesophilic range. In the present research work the concentration of FS inside the anaerobic digester during Stages I and III was close to 50 mg FS-S/L, which is the concentration that provokes the start-up of the inhibition, while during Stage II it was higher (92 ± 49 mg FS-S/L). Despite these high values no inhibition due to the free sulphide was observed throughout Stage II. Among the

microbial populations involved in the anaerobic digestion the methanogenic microorganisms are the most sensitive and the fermentative ones are the less sensitive to sulphide toxicity (Appels et al., 2008). The concentration levels reported in the literature to provoke inhibition are variable. In general, FS concentrations in the liquid phase above 50 mg FS-S/L (at pH 7) are reported to provoke the beginning of toxic issues, causing a reduction as high as 50% in cumulative methane production over a fixed period of exposure time (Chen et al., 2008).

The anaerobic BD values obtained for the brackish sludge in the present study were between 23-42 % (Table 3.2), which are values similar to those obtained during the anaerobic digestion of a waste activated sludge without brackish conditions of 30-50% (Mottet et al., 2010). Specifically, in a previous research work, BD values of 33% for AGS were obtained in batch assays (Val del Rio et al., 2011). In particular, during Stage II, when the reactor was operated under the highest sodium and FS concentrations, no significant inhibition was detected in terms of BD decrease (37-40% BD). However, at this point, the values of sodium and FS reached inside the anaerobic digester were close to the toxic levels for the AD process. Other authors, treating different types of sludge, found that the AD of high saline wastes was feasible and they also studied its limitations related to sodium inhibition, As an example, Jard et al. (2012) found that no relevant effect was detected up to concentrations of 3.1 g Na^+/L in the reactor while treating one specific type of algae (Jard et al., 2012). Another tested saline substrate was sludge collected from a salmon fish farm which allowed the operation of the AD process in stable conditions when diluted sludge containing 5.32 g Na^+/L was used (Gebauer, 2004). Moreover, the AD of sludge has been proved to be feasible even when using NaOH as alkaline pre-treatment, involving relevant concentrations of sodium (Lin et al., 2009). This demonstrates the potential for the minimization of brackish sludge amounts by using AD.

Respect to the composition of the biogas, its methane content ranged between 56 and 62% (Table 3.2). This values are within the expected range of values for sewage anaerobic digestion of 55 – 65% (Appels et al., 2008). However, the high sulphate concentrations provoked the presence of H_2S in the biogas with a maximum value of 3.8% during Stage II which caused problems of corrosion of the metallic parts of the reactor system. The maximum content of H_2S that can be present in the biogas depends on its further use but, in general, concentrations of H_2S in the gas phase over 300 ppm (0.3%) damage the energy conversion machine (Holm-Nielsen

et al., 2009). The values obtained in the present study are above these limit concentrations (between 1.4% and 3.8%). This means that a pre-treatment of the biogas is required to remove the H_2S to avoid corrosion, odour or any other operational problems.

3.5 CONCLUSIONS

The anaerobic digestion of AGS and FLAS under brackish conditions provides similar results in terms of BD with values of 32% and 27% respectively, which demonstrate the feasibility of the anaerobic digestion of an aerobic sludge, independently of the biomass aggregation.

The brackish conditions led to concentrations of sodium and free sulphide near the inhibitory levels reported for anaerobic treatment of wastewater although the BMP and BD measured in this study indicated that neither sodium nor free sulphide had an inhibitory effect in the anaerobic sludge digestion at the studied concentrations. This conclusion was corroborated by the measured SMA, which was the same for the sludge under brackish and non-brackish conditions (0.015 and 0.016 L CH_4 /(g VS · d) respectively). Having this in mind, the low methanogenic activities appears to be more related to the limited hydrolytic activities during solids degradation than to the possible inhibitory effects of the saline conditions.

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Chapter 4
PRODUCTION OF ORGANIC ACIDS FROM FISH-CANNING WASTEWATERS

SUMMARY

The present study explores the feasibility of the production of organic acids using distinct types of fish-canning wastewater as influent for two acidifying reactors. One of the wastewaters comes from the washing of the boilers (WW) and the other is the wastewater produced after boiling the tuna (WC). The main difference between them relays in the fact that the washing effluent has lower organic matter content in comparison with the cooking wastewater (4.16 and 16.3 COD_S for WW and WC, respectively). Also the content in volatile fatty acids (VFAs) (0.26 and 9.27 g COD_{VFA}/L for WW and WC, respectively) makes a difference in terms of anaerobic treatment. Acetic was the most produced acid: 45.5 COD_{VFA}% when acidifying the washing wastewater and 43.1 COD_{VFA}% when acidifying the cooking wastewater when comparing the steady state of both reactors. Nonetheless, propionic acid was also profusely produced during the acidification of WW (27.7 COD_{VFA}%) while, on the other hand, butyric was favoured during the acidification of WC (38.4 COD_{VFA}%), probably due to the high salinity (22.4 ± 4.3 g NaCl/L).

The present study demonstrates the feasibility of acidifying very complex substrates, like fish-canning ones, which are rich in lipids and proteins with the added difficulty of the high NaCl concentrations.

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4.1 INTRODUCTION

The anaerobic digestion (AD) is one of the most spread biological treatment processes for the valorisation of residues due to the operational benefits associated to its biogas production (Lettinga, 1995; Tchobanoglous et al., 2003). In addition, the AD process can provide other value-added products very useful for the chemical industry (Lin et al., 2013), like volatile fatty acids (VFAs). The VFAs are produced during the acidogenesis, which is the second stage of the AD after the hydrolysis. In this stage, the organic acids are produced together with alcohols (Cohen et al., 1979; Husain, 1998). The produced VFAs can be further valorised by the direct use of the effluent or after being recovered from the liquid mixture and purified. The choice between both alternatives depends on their further use. For example, VFA-rich streams can be utilized as substrate for the production of several value-added products like bioplastics, alcohol fuels, biodiesel, bioenergy (electricity through microbial fuel cells, production of hydrogen…) or to carry out biological nutrient removal via denitrification, among other applications (Lee et al., 2014; Silva et al., 2013; Yin et al., 2016). Pure VFAs are widely used in pharmaceutical, textile and plastic industries (den Boer et al., 2016).

There are several established methods for the production of organic acids. Their performances differ from each other since the concentrations, yields and composition of the produced VFAs are affected by the operational conditions (Lee et al., 2014) and the characteristics and composition of the used substrate (Silva et al., 2013). Parameters like the hydraulic retention time (HRT), pH, temperature, type of substrate, C/N ratio in the feeding, and inoculum characteristics, among others, have a manifest influence on VFA production (Yin et al., 2016). With respect to the HRT, high values of this parameter lead to large VFAs concentrations, although methanogens might grow under some operational conditions (Jankowska et al., 2015). For this reason, other parameters need to be controlled as well. As an example, the acidification of papermill wastewater at a constant pH of 6 in a reactor operated at a HRT of 6 hours lead to a yield of acidification of 0.64 g COD_{VFA}/g COD_S, while the increase of the HRT to 24 hours lead to a value of 0.75 g COD_{VFA}/g COD_S (Bengtsson et al., 2008). Values of pH lower than 3 or higher than 12 are inhibitory for the acidogens (Khan et al., 2016; Liu et al., 2012), while the optimal values range between 5 and 11, depending on the type of waste that is being digested (Lee et al., 2014). Furthermore, the pH value also affects the acetate, propionate, butyrate and valerate acids share in the produced effluent. For instance, when increasing the pH from 5.25 to 6.00, the

production of acetate and butyrate decreases while that from propionate augments (Bengtsson et al., 2008). With respect to the temperature, its increase promotes the VFA production and increases their concentration and yield, although, its influence is lower in comparison to that of the pH (Lee et al., 2014). The raise in the temperature from 10 °C to 35 °C increases by 300% the VFA concentration when waste activated sludge is submitted to fermentation (Zhang et al., 2009).

The residues that can be eligible as feedstock for VFA production are abundant. Several solid and liquid wastes have been already studied as potential feedstocks for VFA production like primary and secondary sludge, municipal solid wastes, paper mill effluents, and cheese whey, among many others (Lee et al., 2014; Silva et al., 2013). Nevertheless, it is necessary to consider the type of waste and its composition since they influence the acidifying process (Yin et al., 2016). To the knowledge of the authors, fish-canning wastewaters have not been chosen yet for VFA production although they are eligible for the process due to their content in organic matter. Fish-canning wastewaters are characterised for being very variable in composition depending on the treated feedstock and the manufacture activity. Despite this changeability, they usually have high concentrations of organic matter (0.2 – 55.0 g COD/L) and in many cases of salinity (0.5 – 33.0 g NaCl /L) (Soto et al., 1990). The treatment of this type of effluents has been usually done by means of physico-chemical processes like coagulation, flocculation, filtration, etc. (Lefebvre and Moletta, 2006; Omil, 2003). Nowadays, another feasible option is the treatment of these wastewaters by means of different biological systems, operated in aerobic or anaerobic conditions (Artiga et al., 2008; Figueroa et al., 2008; Omil, 2003; Soto et al., 1993). Considering that these wastewaters can be biologically treated, the production of VFAs is explored throughout this chapter.

The reason for choosing the fish-canning wastewaters is due to the fact that this industrial sector is of key importance to the local economy of the region of the study (Galicia, Spain) and generates huge volumes of liquid effluents that need to be treated. Furthermore, Galician fish-canning industries are also relevant for the Spanish market since they account for the 85% of the economic value of the whole national manufacture and the 87% of the total production volume of this industrial sector (ANFACO-CECOPESCA, 2014). In Europe, the marine industrial sector represents about 5.4 million jobs although more growth is expected (European Commission-Maritime Affairs, 2016). This sector is gaining relevance since it is key for the growth

of the European Union (European Commission-Executive Agency for Small and Medium-sized Enterprises, 2016).

However, several constraints must be considered during the biological treatment of fish-canning wastewaters and, hence, during the VFA production process. Special attention must be paid to their high saline, organic matter content and in some cases proteins concentrations. Hence, the sodium chloride together with high concentrations of ammonia due to protein degradation are expected to be inhibitory since they are problematic for the conventional anaerobic process (Chen et al., 2008).

4.2 OBJECTIVE

The aim of the present study is to investigate the feasibility of using two different tuna processing wastewaters for VFA production. One of them was produced from the washing of the tuna boilers and the other was the tuna boilers effluent. Consequently, the specific objectives of this study are:

- To investigate the VFA production from two different waste streams with variable salinity that can be typically found in the effluents from a fish-canning factory.
- To assess the effects of the composition of the used wastewater on the obtained VFA composition.

These two wastewaters were chosen due to their differences in organic matter content but also in NaCl concentration. The wastewater from the washing of the tuna boilers is characterised by lower saline and organic matter content compared to the wastewater that came from boiling of the tuna filets.

4.3 MATERIALS AND METHODS

4.3.1 Experimental setup

4.3.1.1 Reactor treating the wastewater from the washing of the tuna boilers (RWW)

A sequencing batch reactor (SBR) (Álamo, Spain) with a useful volume of 1.8 L was used for the acidification of the wastewater produced after washing the tuna boilers (Figure 4.2). The SBR was operated at 30 °C using a thermostatic bath (Techne

Inc., USA) so as to maintain the temperature of operation in the mesophilic range. The SBR was operated under complete mixing conditions with a mechanical stirrer at 150 rpm (Heidolph, Germany). The composition of the gas phase in the head-space of the reactor was measured during the first days of operation so as to ensure that no methane was produced.

The reactor operated in 12-hour cycles at a HRT of 1 day. A volume of 0.9 L of influent was supplied per cycle in 15 minutes by using a peristaltic pump (Masterflex, USA). At the end of the cycle the same volume of effluent was withdrawal with another peristaltic pump. The description of the cycle distribution is shown in Figure 4.1. After 18 days of operation, the SBR achieved the steady state in terms of amount of COD transformed into VFAs and VFA composition.

(1) Feeding				
(2) Reaction				
(3) Withdrawal				
(4) Idle				
Time (h)	0.25	11.25	0.25	0.25

Figure 4.1. Time distribution of the phases comprising the operational cycle in the anaerobic SBR.

4.3.1.2 Reactor treating the wastewater from the tuna boilers (RWC)

A semi-continuous stirred tank reactor (Álamo, Spain) with a useful volume of 1.5 L (Figure 4.2) was used for the acidification of the wastewater coming from the tuna boilers. The reactor was operated in the mesophilic range at 35 °C using a thermostatic bath (Techne Inc., USA). It was operated under mixing conditions with a mechanical stirrer at 150 rpm (Heidolph, Germany). The composition of the gas phase in the head-space of the reactor was measured during the first days of operation so as to ensure that no methane was produced.

A volume of about 190 mL was supplied in 15 minutes by a peristaltic pump (Masterflex, USA) every 6 hours due to pump flow limitations. The same amount of effluent was withdrawal simultaneously with the influent addition by liquid overflow.

Figure 4.2. Images of the reactor treating the wastewater from the washing of the tuna boilers (RWW) (left) and the reactor treating the wastewater from the tuna boilers (RWC) (right) configurations.

4.3.2 Operational conditions

Both reactors were inoculated with anaerobic granular sludge from a pilot-scale upflow anaerobic sludge blanket (UASB) treating a synthetic influent mimicking a low-strength wastewater in operation in the laboratories of the Biogroup (Santiago de Compostela, Spain) (Buntner et al., 2011).

4.3.2.1 Characteristics of the fish-canning wastewater

Two different types of fish-canning wastewater were tested as feedstock for VFA production at bench-scale. The first one was the wastewater coming from the washing of the tuna boilers of the factory of Calvo (Carballo, A Coruña, Spain). The second one was collected from the factory of Garavilla, of Conservas Selecta-Isabel (O Grove,

Pontevedra, Spain) and was the withdrawal of the tuna boilers. The compositions of these two wastewaters were significantly different.

Wastewater from the washing of the tuna boilers (WW)

This effluent was characterised by the relatively low concentration of organic matter (Soto et al., 1990) (Table 4.1). The COD_T of the wastewater from the wash of the tuna boilers was 5.36 ± 0.04 g/L while the soluble chemical oxygen demand COD_S was 4.16 ± 0.38 g/L. The BOD_5 (Table 4.1) was 3.18 ± 0.03 g/L, which corresponds to a BOD_5/COD_T ratio of 0.6. This value is representative of an easy treatable wastewater by the use of a biological process (Tchobanoglous et al., 2003).

Table 4.1. Compositions of the wastewater from the washing (WW) and from the boilers (WC).

Measured Parameters	WW	WC
TSS (g/L)	0.54 ± 0.07	2.45 ± 1.17
VSS (g/L)	0.53 ± 0.08	1.84 ± 0.84
VSS/TSS (%)	98.5 ± 0.9	76.1 ± 5.1
BOD_5 (g/L)	3.18 ± 0.03	12.75 ± 0.25
COD_T (g/L)	5.36 ± 0.04	29.9 ± 2.6
COD_S (g/L)	4.16 ± 0.38	16.3 ± 0.9
Fats (g/L)	0.78 ± 0.00	1.18 ± 0.11
Proteins (g/L)	0.76 ± 0.02	1.91 ± 0.41
Carbohydrates (g/L)	$0.12 \pm 3 \cdot 10^{-3}$	0.15 ± 0.03
VFAs (g COD/L)	0.26 ± 0.03	$9.27 \pm 4.5 \cdot 10^{-3}$

To express the concentrations of fats, proteins and carbohydrates (CH) into COD units, the conversion factors used were 2.91 g COD_{FAT} /g; 1.51 g $COD_{PROTEIN}$/g; and 1.07 g $COD_{CARBOHYDRATE}$/g. (Mahmoud et al., 2004). The corresponding COD_S fraction to the each one of them was 2.06 ± 0.18 g COD_{FAT}/L; 1.30 ± 0.03 g $COD_{PROTEIN}$ /L; and $0.12 \pm 3 \cdot 10^{-3}$ g $COD_{CARBOHYDRATE}$/L. The concentration of VFAs was 0.26 ± 0.03 g COD/L. Approximately 6% of the COD_S remained undetermined (Figure 4.3), probably due to measurement errors or maybe due to the possible presence of other organic compounds that were not analysed.

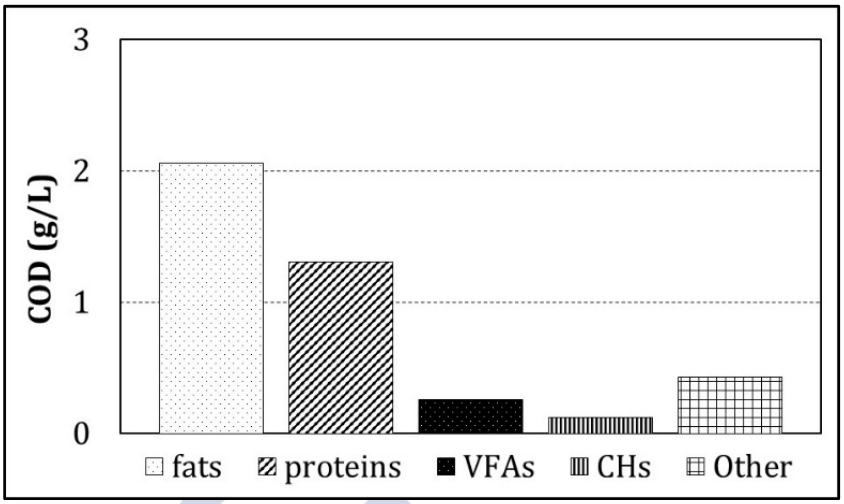

Figure 4.3. Composition of the washing wastewater (WW) expressed as COD (average COD_S: 4.16 g/L).

Wastewater from the tuna boilers (WC)

This wastewater was characterised by the high concentrations of organic matter (Table 4.1). The average COD_T concentration was 29.9 ± 2.6 g COD/L while the COD_S was 16.3 ± 0.9 g COD/L. The BOD_5 was 12.75 ± 0.25 g/L, which corresponds to a BOD_5/COD_T ratio of 0.4. This value is lower in comparison to the BOD_5 of the boilers washing. The corresponding COD_S fraction to the particulate organic matter was 3.36 ± 0.32 COD_{Fat}/L; 3.42 ± 0.49 g $COD_{Protein}$/L; and 0.15 ± 0.03 g $COD_{Carbohydrate}$/L. The VFA content was 9.27 ± $4 \cdot 10^{-3}$ g COD/L. The amount corresponding to each type of compound can be seen in Figure 4.4.

The composition of this second wastewater changed throughout the operational period. After day 96 onwards, the concentration of COD_T was 26.3 ± 3.5 g COD/L while the COD_S of WC was 22.8 ± 3.4 g COD/L due to changes in the operation of the boilers in the factory. For this reason, to establish a generic characterisation of the WC is difficult. Furthermore, the characteristics of the organic compounds themselves did also influence some changes on the composition of the WC throughout time. The lipids floated over the water phase since the wastewater was stored in tanks and the mixing did not exist. Although the wastewater was stored at low temperature, degradation of the organic matter occurred in two ways: (1) during the cooling of the wastewater after its collection from the boilers at hot temperature; and (2) degradation of the

organic matter at low temperature in the tanks. Both wastewaters, with the indicated composition, were filtered to get rid of the pieces from tuna before being supplied to the reactor so as to avoid the clogging of the pumping system but also to have a reliable measurement of the biomass inside the reactor.

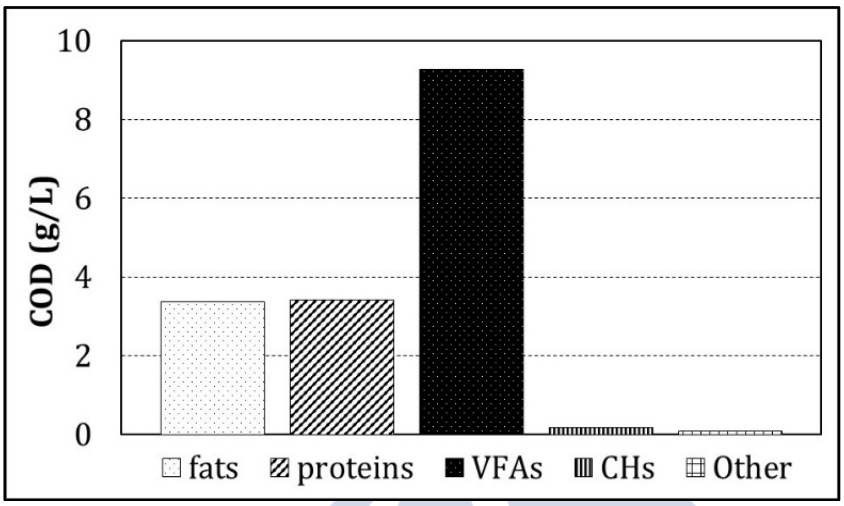

Figure 4.4. Composition of tuna boilers wastewater (WC) expressed as COD (average CODS: 16.3 g/L).

4.3.3 Analytical Methods

The concentrations of Total and Soluble Chemical Oxygen Demand (COD_T and COD_S, respectively) were determined following the method described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005) modified for samples with high concentration of salinity (Soto et al., 1989). The 5-day Biological Oxygen Demand (BOD_5) concentration was determined using the *Oxytop* equipment (WTW, Germany). The VFA concentration and the alkalinity were determined following the method described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). The concentrations of total ammonium (NH_4^+) were determined spectrophotometrically (Bower and Holm-Hansen, 1980) and those from other ions, like sodium (Na^+), potassium (K^+) and sulphate (SO_4^{2-}), by ionic chromatography (IC). The pH was measured with an electrode (52-03, Crison Instruments, USA) and the temperature with a digital thermometer.

Biogas composition was analysed by GC (Hewlett Packard 5890A, USA).

The concentrations of Total (TSS) and Volatile Suspended Solids (VSS) and also the lipids were determined according to the methods described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). The determination of proteins was done according a modified method based on two other methods (Frolund et al., 1995; Lowry et al., 1951). The concentration of carbohydrates was analysed using a modified phenol–sulphuric acid method (Dubois et al., 1956).

All these methods are described in detail in Chapter 2 "Materials and Methods".

4.4 RESULTS AND DISCUSSION

The results obtained from the operation of the acidification reactor treating the two wastewaters tested in the study are presented in this section. The effects of the presence of salt and ammonia nitrogen on the VFAs composition and concentrations were. Moreover, few research studies report results about the VFA production, quantification and characterisation of systems rich in carbohydrates, proteins and lipids (Yin et al., 2016) as it is the case of the present study.

4.4.1 Acidification of the wastewater from the washing of the tuna boilers (RWW)

The reactor treating the wastewater from washing the tuna boilers (RWW) was operated for 60 days at 32 °C, and stirred at 150 rpm to ensure a good mixing and avoid the sedimentation of the biomass. Measured parameters during the reactor operation can be found in Table 4.2. The reactor operated successfully at a pH of 7.2 ± 0.3. At this pH value methanisation would be expected but the measurement of the composition of the biogas indicated that no methane was produced. This could be due to the low solids retention time (SRT), which avoided the proliferation of methanogenic microorganisms, favouring the acidification of the wastewater since SRTs lower than 8 days promote acidogenic conditions (Miron et al., 2000).

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Table 4.2. Average values of the characteristic operational parameters of both reactors during their respective steady states.

Operational Conditions	RWW	RWC				
		RWC1	RWC2	RWC3	RWC4	RWC5
Days of operation (d)	60	0-20	21-34	35-62	63-96	97-397
HRT (d)	1	3	1.5	3	4.5	2
pH	7.2 ± 0.3	7.6 ± 0.3	7.3 ± 0.1	7.8 ± 0.3	7.7 ± 0.3	7.4 ± 0.2
Effluent Composition						
VFA (g COD/L)	3.2 ± 0.2	16.6 ± 0.7	12.8 ± 1.6	15.1 ± 2.5	20.3 ± 1.4	28.6 ± 2.0
NH_4^+-N (g/L)	0.53 ± 0.11	2.1 ± 0.5	2.2 ± 0.3	2.4 ± 0.5	4.0 ± 1.0	4.01 ± 0.83
TSS (g/L)	0.73 ± 0.22	8.4 ± 1.4	5.2 ± 1.2	2.5 ± 0.7	2.2 ± 0.6	2.24 ± 0.52
VSS (g/L)	0.60 ± 0.18	7.3 ± 1.5	4.2 ± 1.1	2.0 ± 0.6	1.6 ± 0.6	1.45 ± 0.38
VSS/TSS (%)	80.9 ± 6.9	86.3 ± 2.0	81.8 ± 2.9	77.6 ± 6.1	73.1 ± 9.5	64.0 ± 5.8
COD_T (g/L)	5.1 ± 0.8	20.0 ± 5.4	19.2 ± 7.3	13.5 ± 1.6	15.3 ± 1.7	32.8 ± 6.4
COD_S (g/L)	4.0 ± 0.1	10.3 ± 1.1	4.8 ± 0.7	14.1 ± 3.7	11.7 ± 2.3	28.5 ± 6.0
Total alkalinity (g/L)	ND	1.13 ± 0.38	1.81± 0.35	7.41 ± 2.65	11.60 ± 0.75	11.40 ± 1.55
Partial alkalinity (g/L)	ND	0.45 ± 0.14	0.62 ± 0.12	2.65 ± 1.09	4.07 ± 0.44	4.34 ± 1.06
NaCl (g/L)	0.95 ± 0.07	5.5 ± 1.0	5.1 ± 0.7	6.4 ± 1.5	16.8 ± 3.6	22.4 ± 4.3
K^+ (g/L)	0.31 ± 0.03	1.3 ± 0.2	1.3 ± 0.3	1.2 ± 0.2	3.9 ± 0.9	1.80 ± 1.11
SO_4^{2-} (g/L)	NDT	1.75 ± 1.04	0.15 ± 0.06	1.0 ± 0.04	0.62 ± 0.09	0.50 ± 0.10

**ND – not determined; NDT: not detected*

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The TSS concentration in the reactor was 0.73 ± 0.22 g/L while the VSS concentration was 0.60 ± 0.18 g/L, which represents about 81% of the TSS. Due to the presence of proteins in the influent, relevant concentrations of ammonium were expected. The NH_4^+-N concentration was 0.53 ± 0.11 g/L. This concentration was not inhibitory for the process although inhibitory values depend on several factors aside the ammonium concentration, like temperature and pH (Chen et al., 2008).

The steady state condition of the reactor operation was identified by a relatively constant concentration (3.2 ± 0.2 g COD_{VFA}/L) and a stable composition of the VFAs in the effluent. This happened approximately after 15 days of operation from the start-up (Figure 4.5). The percentage of organic matter present in the RWW converted into VFAs, in terms of COD, reached a value of approximately 63%. The measured VFAs were acetic (HAc); propionic (HPr); butyric (HBu); and Valeric (HVa) acids. The composition of the different acids was 1.46 ± 0.12 g COD_{HAc}/L; 0.89 ± 0.06 g COD_{HPr}/L; 0.29 ± 0.05 g COD_{HBu}/L; and 0.57 ± 0.04 g COD_{HVa}/L (Figure 4.6). HAc was the main produced acid (45.5 COD%), followed by HPr (27.7 COD%) and n-HVa (17.7 COD%). HBu was the less produced VFA (9.1 COD%).

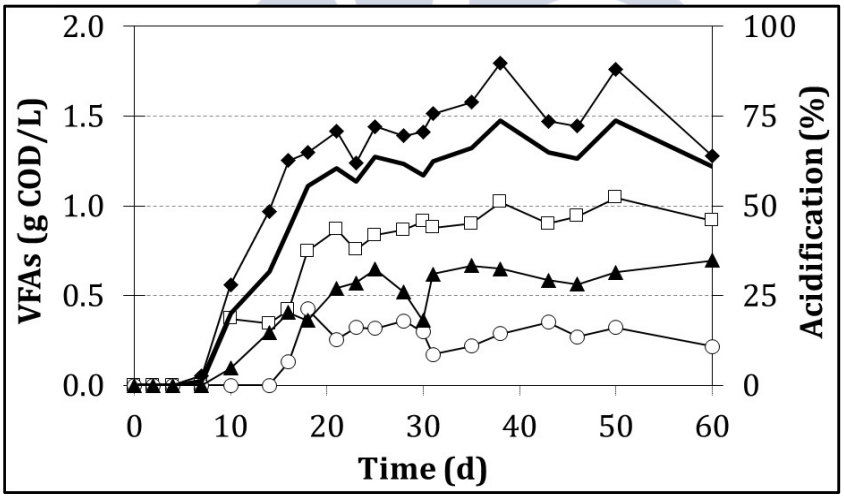

Figure 4.5. Produced VFA concentrations in the reactor treating the washing effluent (RWW): Acetic (◆); Propionic (□); Butyric (○); and Valeric (▲) acids, (g COD/L); and the acidification (%) (-).

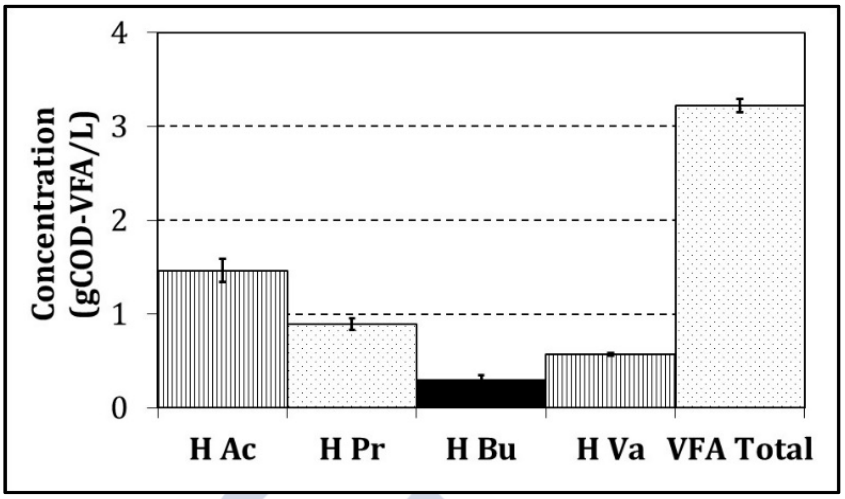

Figure 4.6. Performance of the reactor treating the washing effluent (RWW).

4.4.2 Acidification of the wastewater from the tuna boilers (RWC)

The reactor treating the wastewater from cooking the tuna (RWC), which was directly poured from the tuna boilers, was operated for 397 days at a HRT of 2 days. The reactor was operated in the mesophilic range at a temperature of 35 °C and constantly stirred at 150 rpm, to guarantee a good mixing. Measured parameters during the reactor operation are available in Table 4.2. The large standard deviations of these measured parameters are related to the variability of the composition of the used wastewater. In addition, the wastewater was stored in containers of 25 L, which were difficult to mix before the preparing the feeding. This situation increased the variability of the feeding composition due, for example, to the floatation of fats. For this reason, the presented results were analysed divided in five stages.

The huge concentrations of VFAs in the feeding (Figure 4.4) were accumulated due to the high temperature of operation in the boilers, where the wastewater is produced during tuna cooking. This fact, together with the idle time due to storage, probably induced the degradation of a great amount of organic matter prior to its treatment in the acidifying reactor. The steady state operational conditions were achieved after 6 days of operation and the percentage of transformed COD was of 50% (Figure 4.7). Then new batches of wastewater were collected and the maximum COD conversion increased to approximately 87% and significant changes of the shares of the different VFAs were measured. The determined VFAs during the whole reactor

operation were acetic (HAc); propionic (HPr); butyric (HBu); and valeric (HVa) acids. After reaching the steady state, the composition of the different acids was 12.32 ± 0.94 g COD_{HAc}/L; 3.59 ± 0.92 g COD_{HPr}/L; 10.98 ± 1.37 g COD_{HBu}/L; and 1.68 ± 0.20 g COD_{HVa}/L (Figure 4.8). HAc was the main produced acid (43.1 COD%), closely followed by HBu (38.4 COD%). HPr and HVa were produced too, amounting to 12.6 COD% and 5.9 COD%, respectively.

The TSS concentration in the reactor was 2.24 ± 0.52 g TSS/L while the VSS concentration was 1.45 ± 0.38 g VSS/L, which represents about 64% of the TSS. The VSS/TSS ratio is quite low, indicating that an accumulation of inorganic compounds occurred. This assumption can be corroborated by the high saline content in the reactor (Table 4.2) of 22.4 ± 4.3 g NaCl/L. In these conditions the inhibition of the acidogenic process can occur. Other inhibitory factor is the high concentration of ammonium (4.01 ± 0.83 g NH_4^+-N/L) due to the presence of proteins in the wastewater.

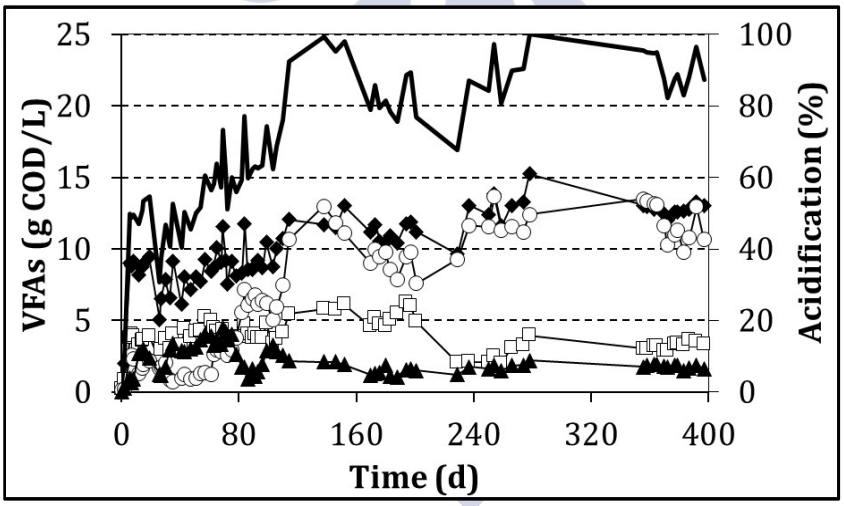

Figure 4.7. Produced VFAs in the reactor treating the effluent of the cookers (RWC): Acetic (g/L) (◆); Propionic (□); Butyric (○); and Valeric (▲) acids, and the acidification (%) (-).

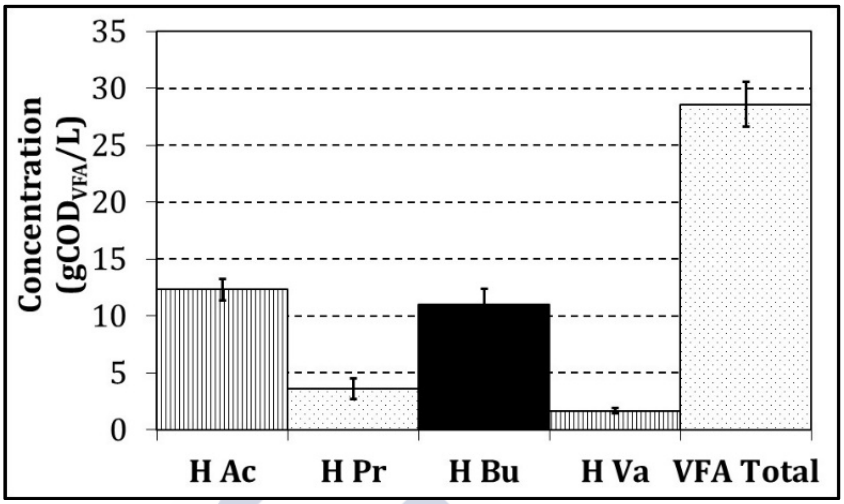

Figure 4.8. Average concentrations of the obtained VFAs in the reactor treating the effluent of the cookers(RWC).

The alkalinity was followed throughout the operation. This parameter is very important since it is an indicator of the neutralizing capacity of the wastewater. In the case of the RWC, a relevant VFA concentration of 9.27 ± 4.5·10^{-3} g COD$_{VFA}$/L was present (Table 4.1) and a larger concentration of 28.6 ± 2.0 g COD$_{VFA}$/L was measured in the effluent of the acidification reactor (Table 4.2). The total alkalinity (TA), due to the contribution of bicarbonate and VFAs, was 11.4 ± 1.6 g/L while the partial alkalinity (PA), due to just the presence of bicarbonate, was 4.3 ± 1.1 g/L.

The pH was 7.4 ± 0.2. Nonetheless, biogas was periodically measured and analysed to ensure that no methane was being produced while most of the organic matter was in the form of VFAs. In addition, the selective production of a volatile fatty acid needs the adjustment of the optimal pH value (Khan et al., 2016). In this study, no optimal pH value was sought because there was no interest in adding chemicals since it increases the operational costs of the process. Consequently, no specific mixture of VFAs was pursued although they were measured and characterised.

4.4.3 VFA composition and NaCl influence

In the present study, the two wastewaters tested were acidified with the aim of characterising the respective reactor operational features and compare the results. These industrial effluents are characterised by their richness in lipids and proteins.

The reactor RWC did not operated at steady state until day 97, while the RWW achieved this state after 6 days. Nevertheless, the composition of the obtained VFAs was marked by the fact that acetic acid was the main obtained product in both reactors (Figure 4.9).

The operation of RWC was separated in four stages in Figure 4.9 so as to show the variations suffered by the VFA composition. These changes are supposed to be due to changes in the feeding composition. The high amounts of HAc produced are due to the high concentration of proteins, since this VFA is the main product of the protein degradation (Yin et al., 2016). Propionic was produced in the same extent for all the stages of RWC while butyric suffered the most important variations During the first 62 days of operation it was the less produced acid while after day 63 it was the second most produced acid. It has been reported that differences in temperature, pH, inoculum, retention time, and/or type of substrate influence the VFA composition (Khan et al., 2016). In the present study, no relevant differences of pH, HRT or temperature were measured. For this reason, observed differences could be attributed to the substrate composition.

When comparing the results from RWW and the steady state (97-397 d) of RWC, it can be seen again that acetic is the most produced acid while relevant differences can be observed in the case of butyric acid. Some authors also observed that higher HRTs lead to the production of longer VFAs (Cavinato et al., 2017), explaining the higher concentration of butyric acid in RWC at a HRT of 2 days while propionic was relevantly produced in RWW at a HRT of 1 day. Another factor that influences the VFA composition in the present study is the substrate composition since it affects the metabolic degradation pathway. For example, HAc is the main VFA for the degradation of proteins while HBu is the principal degradation product of carbohydrates (Yin et al., 2016). Nevertheless, again the type of substrate plays an important role in VFA composition.

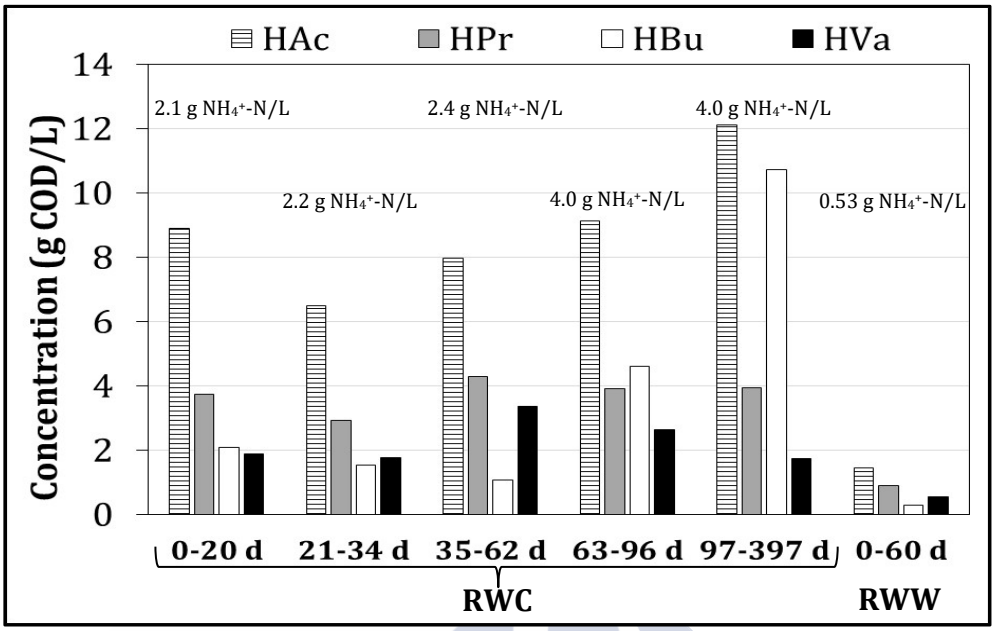

Figure 4.9. Comparison between the VFA composition of the effluents of the reactor treating the washing of the tuna boilers (RWW) and the reactor treating the effluent of the cookers (RWC).

Together with the foreseen parameters affecting the acidification of the fish-canning wastewaters (like HRT, pH, temperature, etc.) the concentration of NaCl should be taken into account. Scarce investigation has been done about the influence of NaCl in the acidification and contradictory results can be found in the literature. On the one hand, the effect of NaCl over the anaerobic fermentation of kitchen waste revealed that the VFA concentration decreased when the salinity of the waste increased. In this case values of 12 g NaCl/L reduced the acidification efficiency to 10% of the in comparison with assays performed in the absence of NaCl (Wang et al., 2014). On the other hand, other authors found that the production of VFAs from waste activated sludge was enhanced under saline conditions and the maximum production of VFAs (3.3 g COD/L) occurred at 29 g NaCl/L (Su et al., 2016). In the present study, the tested salt concentrations did not affect the acidification degree of the substrates since average acidification conversions of 63% and 87% were achieved for WW and WC, respectively. However, this observation might be due to the fact that RWC was operated at concentration of about 5 g NaCl/L during the first 34 days (Figure 4.9) with an acidification percentage of approximately 50 % (Figure 4.7). Concentrations

of 22 g NaCl/L were achieved during the steady state operation of this reactor and the conversion rate increased, achieving acidification percentages usually higher than 80% (Figure 4.7). However, the concentration of NaCl probably affected the VFA composition. Su et al. (2016) found that the increase of NaCl concentrations affected the production of butyric and propionic acids by increasing the production of the first one and decreasing that of the second one. The results obtained in the present study are in accordance with this observation, since higher propionic acid was produced (27.7 COD_{VFA}%) when WW was fed to the reactor and the NaCl concentration was 0.95 ± 0.07 g/L. While butyric acid production (38.4 COD_{VFA}%) was promoted when WC was fed (Figure 4.9) and the NaCl concentration was 22.4 ± 4.3 g NaCl/L.

These effluents rich in VFAs are suitable for different uses like nutrient removal, bioplastic production and bioenergy generation. In the case of nutrient removal, the optimum carbon to nitrogen ratio should be between 5 – 10 g COD/g N to carry out the nitrification-denitrification processes (Lee et al., 2014). Bioplastics (mainly Polyhydroxyalkanoates-PHAs) can be produced by using VFAs from acidified wastes as substrate. PHAs contents conventionally measured are in the range of 20- 65 wt% of PHA using fermented wastes as substrate and mixed microbial cultures as inoculum (Dionisi et al., 2005; Duque et al., 2014; Liu et al., 2008). Even values as high as 77% were achieved when fermented paper mill wastewater was used with a composition of 59 mM of HBu, 49 mM of HAc, 17 mM of propionate and 6 mM of ethanol (Jiang et al., 2012). The use of wastewater as feedstock for biopolymer production would help to reduce the operational cost of the process nearly in one third (Choi and Lee, 1997) with the advantage of being able to produce PHAs with different properties (Palmeiro-Sánchez et al., 2016b). However, acidified fish-canning effluents having concentrations as high as 22.4 g NaCl/L can be inhibitory for this biological process if microorganisms are not adapted to high salinity (Palmeiro-Sánchez et al., 2016a). Finally, waste derived VFAs can be used as source for bioenergy production (biogas, biohydrogen, microbial fuel cells, etc.). Nevertheless, the high ammonia and NaCl contents can be a drawback since in this study concentrations of 4.01 ± 0.83 g NH_4^+-N/L and 22.4 ± 4.3 g NaCl/L were achieved. Ammonium values as high as this one have been found to be inhibitory for methanogens (Chen et al., 2008) or even for biohydrogen production (Lee et al., 2014). Salinity values in terms of NaCl concentrations as high as 22.4 g NaCl/L have been found to be highly inhibitory for biological processes and even the presence of sulphide can be detrimental for the use of these wastes for bioenergy

production since concentrations of 300 ppm in the gas phase damage most of the energy conversion technologies (Holm-Nielsen et al., 2009).

4.5 CONCLUSIONS

This study proves the feasibility of acidifying complex substrates rich in lipids and proteins with the added difficulty of the high NaCl concentrations. As a proof of this, it is remarked that a conversion of 63 % for WW and nearly 87 % for WC regarding the COD was achieved. Nevertheless, the presence of NaCl affected the VFA composition as high saline conditions promote the production of butyric acid.

Despite the high acidification percentages, these effluents would not be suitable for bioenergy production due to process constraint because of the high ammonium and NaCl concentrations. Another option would be their use for biopolymer production since VFA concentrations are suitable for this type of process.

4.6 REFERENCES

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Chapter 5
VERSATILE PRODUCTION OF PHAs WITH MIXED CULTURES AND DIFFERENT SUBSTRATES

SUMMARY

The ability of a microbial mixed culture (MMC), enriched in *Thauera* and *Azoarcus* genera, to produce polyhydroxyalkanoates (PHAs) from different volatile fatty acids (VFAs) mixtures is explored. The MMC was enriched by applying a feast/famine regime and fed with a mixture of VFAs containing 1.52:0.61:0.15:0.25 g/L of acetic (HAc), propionic (HPr), butyric (HBu) and valeric (HVa) acids, respectively. The maximum accumulation capacity of the MMC remained almost constant (39.0-48.4 wt%) with all tested VFA compositions. Although the PHA composition varied significantly (2-76 HV content in PHA%). Slight differences were detected in the obtained biopolymer yields and biopolymer production and substrate uptake rates attributed to the different substrate fed. Furthermore, a comparison between obtained and literature results indicated that if the F:M ratio in the accumulation experiment remains between 1 and 7 Cmol VFA/(Cmol X · cycle) the maximum PHA accumulation yield of approximately 0.8 Cmol PHA/Cmol VFA can be obtained.

The obtained average yield for PHA production using the VFA mixture of the feeding was 0.757 ± 0.093 Cmol PHA/Cmol VFA. Other assayed substrates were HAc, HPr, HBu and HVa. The average yield for these experiments was 0.742 ± 0.019 Cmol PHA/Cmol HAc, 0.683 ± 0.096 Cmol PHA/Cmol HPr, 0.705 ± 0.010 Cmol PHA/Cmol HBu and 0.838 ± 0.082 Cmol PHA/Cmol HVa, respectively. These results indicate that it is feasible to enrich a mixed microbial community able to achieve similar production efficiency when using different VFA in the accumulation stage.

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5.1 INTRODUCTION

Polyhydroxyalkanoates (PHAs) are naturally occurring organic compounds that have been deeply studied during the last years because of their usefulness. They have several applications as: bioplastic, biofuel, medical materials, fine chemicals, etc (Chen, 2010). PHAs can be considered as the only fully bio-based and biodegradable plastic materials because they are directly produced by microorganisms, they are fully biodegradable and can be made from 100% renewable resources (Dias et al., 2006).

Nowadays, extensive research is focused on the PHAs production by microbial mixed cultures (MMC) using wastes as carbon source. Most of these processes operate in two stages. In the first one, PHA accumulating MMC are enriched in sequencing batch reactors (SBR) by applying a "feast-famine" sequence (presence/absence of carbon compounds) leading to periods of carbon source limiting conditions (Albuquerque et al., 2011; Dionisi et al., 2005; Duque et al., 2014; Johnson et al., 2009; Moralejo-Gárate et al., 2011). In the second one, the maximization of biopolymer accumulation inside the cells takes place by the addition of excess of carbon source in a fed batch unit. It seems that the best performance, in terms of PHA production rates and yields, is achieved by accumulation experiments performed with the same substrate as that used for the selection of the used MMC, where PHA accumulation values as high as 89 wt% can be obtained (Johnson et al., 2009). The used substrate significantly defines the physico-chemical properties of the obtained PHAs (Palmeiro-Sánchez et al., 2016). Currently, tailoring biopolymers besides the study of the effect of the substrate composition are at their very beginning and it would be of interest for setting up a waste-based PHA production process (Moralejo-Gárate et al., 2014).

Basic knowledge is already available with respect to the fact that volatile fatty acids (VFAs) with odd number of carbons lead to the production of polyhydroxybutyrate (PHB) but also polyhydroxyvalerate (PHV) and, when using VFAs with even number of carbons, only PHB is stored inside the cells (Lemos et al., 2006). In this way, the substrate determines the physico-chemical properties of the polymer as it influences the monomer distribution (Palmeiro-Sánchez et al., 2016) and, as a consequence, the further application of the obtained biopolymers.

Up to now, few studies have reported on the substrate versatility for PHA production. Lemos et al. (2006) carried out accumulation experiments with two different MMCs: one enriched with propionate and another with acetate. On one hand, these authors found that the accumulation yields of the acetate MMC were similar for propionate and for acetate, with values of 0.82 and 0.88 Cmmol PHA/Cmmol Substrate, respectively. On the other hand, they found that the accumulation yields of the propionate MMC were lower for propionate than for acetate, with values of 0.53 and 0.77 Cmmol PHA/Cmmol Substrate, respectively (Lemos et al., 2006). Chang et al. (2012) performed similar assays with results which were contrary to Lemos et al. (2006). These authors found that the use of propionate for the PHA-accumulation with the MMC enriched with acetate provided low values of approximately 0.15 Cmmol PHA/Cmmol Substrate, while this value was about 0.30 Cmmol PHA/Cmmol Substrate when using acetate as sole carbon source. In contrast, when they used acetate as substrate for the accumulation with a MMC enriched in propionate, this value was close to 0.45 Cmmol PHA/Cmmol Substrate although it was lower than the accumulation with propionate, with a value close to 0.60 Cmmol PHA/Cmmol Substrate. The highest value was achieved when using valerate as substrate with the propionate-enriched MMC, obtaining a value near to 0.7 Cmmol PHA/Cmmol Substrate (Chang et al., 2012). Lemos et al. (2006) also used valerate and butyrate as substrate with the acetate MMC obtaining yields of 0.77 and 0.80 Cmmol PHA/Cmmol Substrate, respectively, which were similar to the one obtained with propionate which was of 0.82 Cmmol PHA/Cmmol Substrate.

When using substrates different from VFAs, the sole study performed until now with MMCs was performed by Moralejo et al. (2014). These authors enriched a MMC with commercial glycerol and studied the accumulation with glucose, lactate, acetate, glycerol and xylitol. Positive results for the accumulation with all the substrates tested with exception of xylitol were obtained. The amount of biopolymer (PHB together with polyglucose with different ratios) was about 50% for acetate or glycerol while it was about 70% when glucose or lactate was fed. Xylitol was used neither for accumulation nor growth. The reached yields were 0.36 Cmmol PHB/Cmmol Lactate; 0.57 Cmmol PHB/Cmmol Acetate; 0.72 Cmmol BioPolymer/Cmmol Glucose; and 0.77 Cmmol BioPolymer/Cmmol glycerol, indicating the MMC dependence of the substrate (as indication: the Cmmol of Biopolymer are referred to the accumulation of PHB and Polyglucose together).

Taking into account the wide differences on the obtained results using VFAs as carbon source (Chang et al., 2012; Lemos et al., 2006), further research is required to clarify the factors affecting the PHA accumulation capacity of MMC using substrate compositions different from those used during the enrichment. A possible option to widen the versatility of the MMC could be selecting the community in a rich variety of VFAs to diversify the composition of the obtained mixed culture.

5.2 OBJECTIVES

The aim of the present study is to investigate the feasibility of PHA production using different substrates during the accumulation stage since the usual proceeding is to use the same substrate for the enrichment and the accumulation stages. Accordingly, the objectives of this study are:

- To evaluate the accumulation of biopolymer when using different substrates
- To study the effect of substrate versatility over the kinetic and stoichiometric parameters
- To investigate the effect of F/M ratio on PHA accumulation

5.3 MATERIALS AND METHODS

5.3.1 Experimental set-up

Two reactors were used: a SBR for the selection of the enriched microbial mixed culture (MMC) and a fed batch reactor for the accumulation experiments (Table 5.1).

In both cases the pH value was not controlled but it was monitored on-line with a pH-meter equipped with a membrane electrode (Crison, Germany). The dissolved oxygen concentration (DO) was measured and acquired with an oxygen meter provided with a membrane sensor (Hach-Lange, USA). The temperature inside the reactors was controlled at 30 °C by means of a thermostatic bath which pumped water through the jackets of both reactors (Techne Inc., USA).

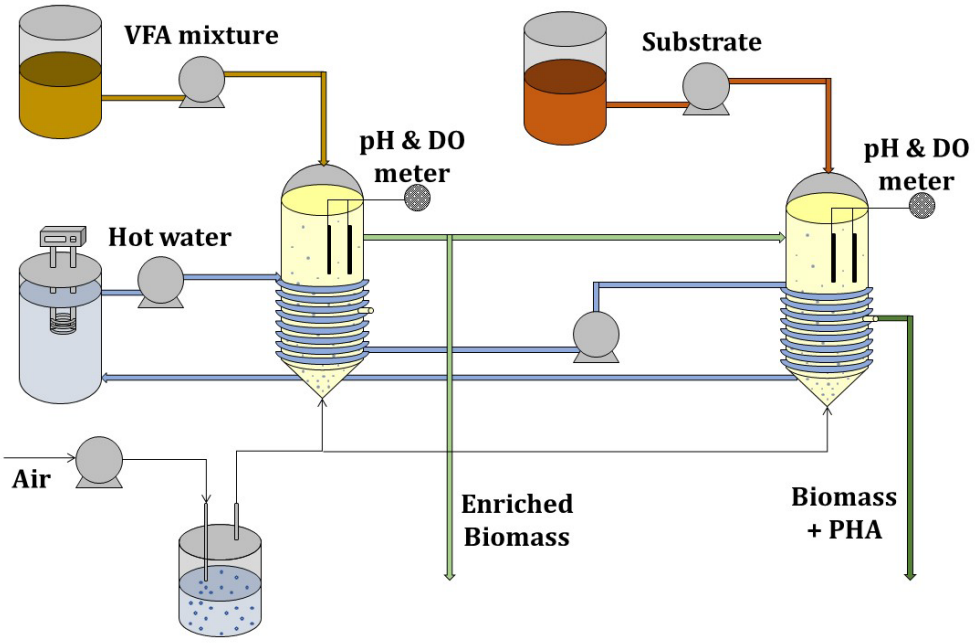

Figure 5.1. Set-up of the PHA production system comprising the enrichment and the accumulation reactors.

5.3.1.1 Enrichment Reactor

A SBR (Álamo, Spain), with a working volume of 1.8 L, was used for the selection and production of the accumulating microorganisms. The pH value was measured on-line and registered at the end of the famine phase with a membrane electrode (Crison, Germany). Air was supplied to the reactor through an air diffusor and the dissolved oxygen (DO) concentration was measured and acquired with an oxygen meter provided with a membrane sensor (Hach-Lange, USA). The reactor was jacketed, so the temperature was maintained at 30 °C with a thermostatic water bath (Techne Inc., USA).

5.3.1.2 Accumulation Reactor

A fed-batch reactor (Álamo, Spain) was used to evaluate the PHA accumulation capacity of the previously enriched MMC. This reactor was inoculated with the biomass freshly harvested at the end of one operational cycle of the SBR.

The pH value was not controlled although it was measured with a membrane electrode (Crison, Germany) throughout the accumulation experiment. The DO concentration was measured and acquired during the entire accumulation assay by using an oxygen meter provided with a membrane sensor (Hach-Lange, USA). The reactor was jacketed and its temperature was about 30 °C by means of a thermostatic bath (Techne Inc., USA).

5.3.2 Operational Conditions

5.3.2.1 Enrichment Reactor

The reactor was inoculated with 1.8 L of activated sludge at a concentration of 0.46 g VSS/L and collected from a wastewater treatment plant (Calo-Milladoiro, Spain). The SBR was operated in 12-hour cycles distributed in: feeding (15 min); reaction (675 min); withdrawal (15 min) and idle phases (15 min). Then, an aerobic dynamic feeding strategy was applied to impose the feast and famine periods required for the culture selection. The SBR was operated under non-sterile conditions and without pH control. The hydraulic and solids retention times were fixed at 24 h.

The SBR was operated for 1050 days at an organic loading rate of 3.33 g $COD_{VFA}/(L \cdot d)$. The exchanged volume of reactor media was of 0.9 L per cycle and at the beginning of each cycle a mixture of carbon and nutrient source solutions (1:1) was added. The added volume of carbon source solution was of 0.45 L, containing 1.52/0.61/0.15/0.25 g/L of acetic (HAc), propionic (HPr), butyric (HBu) and valeric (HVa) acids, respectively. The volume of nutrients medium was also of 0.45 L and it comprised: NH_4Cl 0.66 g/L, KH_2PO_4 0.35 g/L, $MgSO_4 \cdot 7\ H_2O$ 0.14 g/L, KCl 0.05 g/L, 1.5 mL of trace elements solution (Vishniac and Santer, 1957) and 0.15 mL/L of a solution of 33 g/L of allylthiourea (ATU). ATU was added to prevent the nitrification process to take place.

The performance of the enrichment reactor was monitored twice a week. By measuring pH, VSS, TSS, and ammonium concentrations at the end of the famine phase. Enrichment cycles were performed to evaluate the progression of VFAs, VSS, TSS, ammonium and PHA concentrations throughout the SBR cycle. Samples were taken at different times. The obtained data was used to calculate the stoichiometric and kinetic parameters corresponding of the enrichment.

5.3.2.2 Accumulation Reactor

The accumulation reactor was inoculated with biomass withdrawn from the enrichment reactor at the end of the SBR cycle. Certain amount of VFAs (Table 5.1) was supplied in pulses of carbon source solution added every time the oxygen concentration in the reactor rose up, which meant that the previously added substrate pulse was totally consumed. In this way, the reactor operated under excess of organic matter. The pH value was not controlled although it was measured with a membrane electrode (Crison, Germany) throughout the accumulation experiment. The DO concentration was measured and acquired during the entire accumulation assay with an oxygen meter provided with a membrane sensor (Hach-Lange, USA).

Accumulation experiments were performed to evaluate the maximum accumulation capacity of the MMC and to determine the stoichiometric and kinetic parameters obtained with different substrates at the same temperature. The temperature was controlled at 30 °C by using a thermostatic bath (Techne Inc., USA).

Five compositions were evaluated containing single HAc, HPr, HBu, HVa, and the mixture of VFAs (HAc:HPr:HBu:HVa) used for the enrichment stage (Table 5.1). All the accumulation experiments were performed under the same operational conditions of the enrichment reactor. It was operated under non-sterile conditions.

No nitrogen source was supplied so as to prevent the biomass growth. A small amount of N was present at the beginning of the experiment contained in the bulk liquid accompanying the biomass collected from the enrichment SBR.

Table 5.1. Feeding composition of the batch accumulation assays.

Feeding	VFA Composition (COD%)	Pulse (Cmmol VFA/g VSS)
HAc	100:0:0:0	6.1 ± 2.1
HPr	0:100:0:0	12.9 ± 5.5
HBu	0:0:100:0	16.1 ± 6.8
HVa	0:0:0:100	12.6 ± 0.3
VFA mixture	49:28:8:15	8.2 ± 0.7

5.3.3 Analytical Methods

Liquid samples taken from the reactors were filtered with a mixed cellulose ester filter of 0.45 µm pore size (Advantec, Japan). VFA concentrations were measured following a the method described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005) by gas chromatography (GC) (Hewlett Packard 5890A, USA). The concentrations of total ammonium (NH_4^+) were determined spectrophotometrically (Bower and Holm-Hansen, 1980). pH was measured with an electrode (52-03, Crison Instruments, USA). The temperature was determined with a digital thermometer. The DO concentration was measured and acquired with an oxygen pocket meter (Hach-Lange, USA).

The concentrations of Total (TSS) and Volatile Suspended Solids (VSS) were determined according to the methods described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). PHA was extracted and analysed by a chromatographic method (Smolders et al., 1994).

The characterization of the microbial populations was performed by the FISH technique (Amann et al., 1990). The hybridization step was performed with the following probes: EUB338mix for Bacteria, AZO644 for *Azoarcus*, CTE for *Comamonas*, MZ1 for *Thauera*, GAM42a for *Gammaproteobacteria* and PAR1244 for *Paracoccus*. The abundance of these populations was tested by the Daime software (Daims et al., 2006).

All these methods are described in detail in Chapter 2 “Materials and Methods”.

5.3.4 Calculations

Calculations related to kinetic and stoichiometric values for PHA production and carbon balances, the amount of PHA, active biomass, CO_2, rates and also the yields for each produced compound were determined in each enrichment and/or accumulation assay. All these calculations are described in detail in Chapter 2 “Materials and Methods”.

5.4 RESULTS AND DISCUSSION

5.4.1 Enrichment of a MMC with a mixture of VFAs

A MMC was enriched in a SBR, from activated sludge, using a mixture of VFAs (1.52:0.61:0.15:0.25 g/L of HAc:HPr:HBu:HVa) as carbon source. The reactor operated for 1050 days and once the enrichment was achieved the average length of the feast phase lasted for 110 ± 24 min, which represents the 15% of the total cycle length. This percentage is lower than the value of 20%, which was identified as an indicator of the presence of a mixed community with high accumulating capacity (Dionisi et al., 2007; Moralejo-Gárate et al., 2013). Under these conditions, the pH value of the effluent was approximately 9.2 ± 0.3. The concentration of Na^+ was 795 ± 101 g/L due to the addition of NaOH used for neutralizing the acid pH of the feeding. The concentration of TSS was 1.0 ± 0.3 g/L and that of VSS was 0.9 ± 0.3 g/L with a ratio VSS/TSS of 85.6 ± 9.3 %, measured at the end of the enrichment cycle. The NH_4^+-N concentration in the effluent was 27.5 ± 10.5 g/L.

The kinetic and stoichiometric parameters of the enriched MMC were estimated by monitoring several cycle measurements distributed along the 1050 days of operation. Average values of the VFA specific consumption and PHA specific production rates were 0.692 ± 0.183 Cmol VFA/(Cmol X · h) and 0.217 ± 0.094 Cmol PHA/(Cmol X · h), respectively. The individual specific production rates for each measured monomer were 0.106 ± 0.052 Cmol HB/(Cmol X · h) and 0.112 ± 0.062 Cmol HV/(Cmol X · h). The average specific biomass growth rate was 0.226 ± 0.040 Cmol VSS/(Cmol X ·h) and the CO_2 specific production rate was 0.281 ± 0.058 Cmol CO_2/(Cmol X · h). The yield corresponding to the polymer production had an average value of 0.308 ± 0.105 Cmol PHA/Cmol VFA corresponding to the individual yields for each monomer that were 0.154 ± 0.075 Cmol HB/Cmo lVFA and 0.154 ± 0.061 Cmol HV/Cmol VFA. The yield for the biomass production was 0.336 ± 0.062 Cmol X/Cmol VFA and for the CO_2 was 0.413 ± 0.058 Cmol CO_2/Cmol VFA. The average error from the performed mass balances was 5.7%.

5.4.2 Effect of the substrate composition on PHA accumulation

Fed-batch assays were performed to estimate the maximum accumulation capacity of the MMC using different VFAs compositions as substrate (Table 5.2). Operational conditions were the same as in the enrichment reactor. However, the

substrate was added using a pulse-feeding strategy. The concentration and composition of the added VFAs are described in Table 5.1. The average values of the kinetic and stoichiometric parameters were estimated from the results of the experiments (Table 5.3).

Table 5.2. Summary of the average PHA contents and solids concentrations obtained on the accumulation experiments.

Substrate	HB (wt%)	HV (wt%)	total (wt%)	HB:HV (g/g)	TSS (g/L)	VSS (g/L)
HAc	46.50 ± 8.43	1.04 ± 0.38	47.53 ± 8.80	50.55 ± 15.1	3.97 ± 0.86	3.01 ± 0.74
HPr	11.53 ± 2.44	30.76 ± 9.04	42.29 ± 8.67	0.43 ± 0.15	3.11 ± 1.22	2.17 ± 0.72
HBu	38.39 ± 6.05	0.61 ± 0.11	39.00 ± 6.16	63.19 ± 1.49	3.43 ± 0.08	3.24 ± 0.11
HVa	11.72 ± 0.40	36.68 ± 0.61	48.40 ± 1.02	0.32 ± 0.01	3.19 ± 0.33	2.89 ± 0.32
VFA mixture	29.28 ± 4.73	17.87 ± 8.16	47.14 ± 9.82	2.02 ± 0.75	3.03 ± 1.27	2.13 ± 0.66

Table 5.3. Summary of average stoichiometric and kinetic parameters obtained from the accumulation assays.

Substrate	$-q_{VFA}$	q_{HB}	q_{HV}	q_{PHA}	q_{CO2}	Y_{HB}	Y_{HV}	Y_{PHA}	Y_{CO2}
	(Cmol/Cmol X · h)					(Cmol/Cmol VFA)			
HAc	0.357 ±0.059	0.260 ±0.036	0.004 ±0.002	0.264 ±0.039	0.094 ±0.013	0.731 ±0.019	0.011 ±0.005	0.742 ±0.019	0.264 ±0.009
HPr	0.275 ±0.039	0.044 ±0.009	0.140 ±0.031	0.184 ±0.022	0.074 ±0.011	0.166 ±0.040	0.517 ±0.110	0.683 ±0.096	0.269 ±0.005
HBu	0.300 ±0.064	0.210 ±0.047	0.003 ±0.002	0.213 ±0.048	0.078 ±0.021	0.697 ±0.006	0.008 ±0.003	0.705 ±0.010	0.257 ±0.015
HVa	0.346 ±0.039	0.061 ±0.017	0.233 ±0.045	0.294 ±0.061	0.113 ±0.020	0.172 ±0.028	0.666 ±0.054	0.838 ±0.082	0.323 ±0.020
VFA mixture	0.439 ±0.164	0.196 ±0.064	0.142 ±0.098	0.338 ±0.151	0.127 ±0.050	0.462 ±0.059	0.295 ±0.105	0.757 ±0.093	0.290 ±0.031

The influence of the substrate composition on the obtained polymer has not been fully unravelled yet, although a number of studies about PHA accumulation in MMCs using VFAs as carbon source have been reported in the literature. On the one hand, the establishment of a defined carbon source feeding for a certain MMC should provide a constant configuration and distribution of the biopolymer monomers (Albuquerque et al., 2007; Dias et al., 2008). Furthermore, Lemos et al. (2006) have reported that VFAs with even number of carbons in their molecules lead to HB production, while VFAs with odd number of carbons lead to the presence of HB and HV monomers in the biopolymer chains. But, on the other hand, the same substrate composition has been found to lead to different HB:HV distributions depending on the specific characteristics of the enriched microbial community (Albuquerque et al., 2013; Carvalho et al., 2014). For these reasons variable ratios of HB and HV have been reported by several different authors even when using the same substrate (Chang et al., 2012; Dionisi et al., 2004; Lemos et al., 2006). To give some more information about this matter, in the present study several fed-batch assays were performed using different VFAs (Table 5.1) as substrate to test the versatility of the enriched MMC. This versatility is focused on evaluating the amount of accumulated PHA and also on the HB:HV ratios of the obtained biopolymer. However, it should be considered that both parameters do not exclusively depend on the substrate composition, as it has been seen before.

Firstly, the obtained results indicated that the average PHA content (Table 5.2) for each assayed substrate was 47.53 ± 8.80 wt% for HAc, 42.29 ± 8.67 wt% for HPr, 39.00 ± 6.16 wt% for HBu, 48.40 ± 1.02 wt% for HVa and 47.14 ± 9.82 wt% for the VFA mixture. Chang et al. (2012) obtained very satisfactory results for a MMC enriched in propionate with values close to 49 wt% of PHA with acetate and 60 wt% of PHA with valerate. Nevertheless, these values were not so good when a MMC enriched in acetate was used: they obtained 60 wt% of PHA with acetate but just 20wt% of PHA when propionate was fed. These dissimilarities might be attributed to differences in the MMC selection during the enrichment stage, as the substrate was the same but the operational conditions were different. However, it seems that a MMC selected with a feeding comprising several VFAs, like the one presented in this study, can stand better the changes in the substrate during the accumulation without being so affected in terms of PHA production.

Secondly, remarkable differences among the produced biopolymer composition were observed. Particularly, the HV content of the accumulated biopolymers ranged

between 0.61 and 36.68 wt% (Table 5.2), as the compositions of the supplied substrates (Table 5.1) were very different among them. These values represent between 1.68 mol% and 77.09 mol% of HV in the biopolymer when butyrate and valerate were fed, respectively. A value of 2.35 mol% was obtained when acetate was fed. Chang et al. (2012) obtained HV contents of 2 mol% when acetate was fed and 89 mol% when valerate was fed. At low HV content differences are not remarkable but when its presence is relevant, high differences can be detected when using the same substrate. Values of 77 mol% in contrast to 89 mol% of HV where obtained for two different enriched MMCs when using just valerate as substrate (Chang et al., 2012). As indicated before, these different results could be due to the influence of the MMC enrichment stage and the selection of the communities involved in each case. Even so it seems clear that valerate is the preferred substrate for HV production, as observed from both studies.

From the results obtained in the present accumulation assays it seems clear that the average total PHA content in the biomass was not significantly influenced by the substrate composition whereas the accumulated HV ranged from 0.61 to 36.68 wt%, allowing for different copolymers. These two facts lead to the production of different biopolymers from the same enriched biomass, widening the spectrum of the potential applications of the further obtained bioplastics. It is important to remark that tailoring biopolymers can be done by just changing the substrate during the accumulation assays but considering that the enrichment should be in steady state, as the MMC composition also influences the monomer distribution.

5.4.3 Effect of substrate on the kinetic parameters

The specific substrate uptake rate ranged from 0.439 ± 0.164 Cmol VFA/(Cmol X · h) to 0.275 ± 0.039 Cmol VFA/(Cmol X · h) when the VFA mixture and propionic acid were added to the reactor respectively (Table 5.3). The uptake and production rates differ for the single VFAs and the best results were observed for the VFA mixture, since the MMC was enriched with this substrate and therefore adapted to it.

The HV production rates were 0.140 ± 0.031 and 0.233 ± 0.045 Cmol HV/(Cmol X · h) for assays with HPr and HVa, respectively (Table 5.3), with average specific substrate uptake rates of 0.275 ± 0.039 Cmol HPr/(Cmol X · h) and 0.346 ± 0.039 Cmol HVa/(Cmol X · h), respectively. This different behaviour between these two substrates could be explained by the fact that propionate is subjected to

decarboxylation to form acetyl-CoA (Fradinho et al., 2014), which is the rate limiting stage (Dias et al., 2008), but valerate just needs to enter the ß-oxidation to form the acetyl-CoA (Chang et al., 2012). This fact could explain the faster consumption of HVa and, subsequently, the faster production of HV in comparison with HPr, as observed in the present study and also in other studies like Chang et al. (2012). It looks as if valerate was the preferred substrate when it refers to the obtainment of high HV production rates.

The HB production rates were similar for the VFAs with even number of carbons, with values of 0.260 ± 0.036 Cmol HB/(Cmol X · h) and 0.210 ± 0.047 Cmol HB/(Cmol X · h) for assays with HAc and HBu, respectively (Table 5.3). The average specific substrate uptake rates were also similar, with values of 0.357 ± 0.059 Cmol HAc/(Cmol X · h) and 0.300 ± 0.0640 Cmol HBu/(Cmol X · h). This behaviour is opposite to that observed by Marang et al. (2013), as they stated that HBu was the preferred substrate for PHB production, even more than acetate. During their investigation, these authors compared the PHB production between two cultures enriched with HBu and HAc:HBu (1:1 Cmol/Cmol) to another MMC enriched with HAc (Jiang et al., 2011). They observed similar rates for the accumulations where butyrate was present (3.64 and 3.72 Cmol HBu/(Cmol X · h)), while values obtained for the HAc accumulation were 1.74 Cmol HAc/(Cmol X · h), which was nearly half of the values obtained with HBu (Jiang et al., 2011; Marang et al., 2013). It must be indicated that the production rates from these studies from the literature were calculated as the maximum values while those from the present research work are average values, so all numeric values cannot be compared rigorously.

At the sight of the previous results, it is clear that the substrate used for the enrichment is preferred by the MMC in terms of substrate uptake and biopolymer production rates. Nevertheless, the community is able to satisfactorily accumulate with different kinetic values in spite of the type of VFA fed to the MMC as it also depends on the substrate concentration (Serafim et al., 2004).

5.4.4 Effect of substrate on the stoichiometric parameters

The yield values obtained for each batch accumulation assay are very useful as they indicate the amount of fed substrate diverted for biopolymer production. In the case of using acetate as substrate for the accumulation, an average production yield of 0.742 ± 0.019 Cmol PHA/Cmol HAc was obtained in the present study. This value

is significantly higher than those found in the literature. A production yield of 0.5 Cmol PHA/Cmol HAc was obtained when acetate was fed to a MMC enriched in this acid (Serafim et al., 2004). Also yields between 0.25 and 0.46 Cmol PHA/Cmol HAc (Chang et al., 2012); between 0.61 and 0.68 Cmol PHA/Cmol HAc (Jiang et al., 2011); and of 0.60 Cmol PHA/Cmol HAc (Johnson et al., 2009) were obtained by other authors for different accumulations with acetate as substrate. Having a look at all these results and referring just to the process of turning substrate into biopolymer, it seems that the yield obtained in the present study confirms a better performance of the MMC obtained by the enrichment with a VFA mixture when it is compared to other different enrichments. A similar behaviour was observed when propionic acid was used as substrate for the accumulation assays.

The average obtained yield in this study was 0.683 ± 0.096 Cmol PHA/Cmol HPr, which is higher than values obtained by other authors. For example, values of 0.23 Cmol PHA/Cmol VFA (Lemos et al., 2006) and about 0.60 Cmol PHA/Cmol HPr (Chang et al., 2012) were obtained when propionate was fed to a MMC enriched also with this substrate. In the case of valerate, a similar trend was followed as the yields obtained with the MMC of this study seem to be better than values obtained by other authors. In this study, an average yield of 0.838 ± 0.082 Cmol PHA/Cmol HVa was reached when feeding HVa to the MMC adapted to a VFA mixture whereas lower values were obtained by other authors. They achieved yields of 0.38 Cmol PHA/Cmol HVa when using a MMC enriched with HAc (Lemos et al., 2006) and 0.71 Cmol PHA/Cmol HVa when using a MMC enriched with HPr (Chang et al., 2012).

On the other hand, butyrate showed a very good yield although it did not show the best reported performance. Marang et al. (2013) achieved a yield of 0.89 Cmol PHA/Cmol HBu while, in this study, the yield was of 0.705 ± 0.010 Cmol PHA/Cmol HBu. Nonetheless, the main difference between both yields is that the value presented by Marang et al. (2013) was determined over the first 4.5 h of the accumulation experiment while the value for this study was the average of the whole accumulation assay. However, to the best of our knowledge, these are the highest achieved yields with butyrate fed to a MMC enriched on another substrate. For example, Lemos et al. (2006) obtained a yield of 0.45 Cmol PHA/Cmol HBu when butyrate was fed to a MMC enriched in acetate.

It can be seen that obtained yields are generally better for all the substrates tested in the present study when they are compared with the results from other

authors in terms of changes in substrate during the accumulation. At the sight of the obtained values, it can be asserted that it is feasible to produce different biopolymers using the same enrichment just by changing the substrate for the accumulation (Table 5.2).

5.4.5 Effect of the F/M ratio on PHA accumulation

From previous experiments, performed to evaluate the accumulation capacity of MMCs using different VFAs, it was stated that the yield of biopolymer accumulation depends mainly on the fed substrate and on the microbial selected population (Lemos et al., 2006). However, no author has paid attention to the effects of the fed to microorganism ratio (F/M ratio). Regarding this, results from other authors studying the accumulation capacity of MMCs under different carbon limiting conditions were analysed (Figure 5.2). A certain trend has been observed indicating that the higher the amount of carbon supplied with respect to the active biomass in the system the higher the amount of PHA accumulated for those F/M values corresponding to limiting carbon conditions (under approximately 1.0 Cmol VFA/(Cmol X · cycle)). Once non-limiting conditions are achieved the maximum accumulating capacity of the biomass can be feasibly reached.

VFA concentration must be restricted to values lower than 37.5 Cmol PHA/(Cmol X · cycle) to avoid the appearance of inhibitory conditions (Serafim et al., 2004). This value corresponds in the research work of Serafim et al. (2004) to a concentration of 180 Cmmol VFA/L of HAc. These authors defend that the application of the feeding in pulses increases the amount of total PHA accumulated inside the biomass. Although, from the results of Albuquerque et al. (2011) using acidified molasses as substrate, it was observed that the use of a continuous feeding strategy allowed obtaining higher substrate uptake and biopolymer storage rates than applying an aerobic dynamic feeding strategy. Furthermore, this continuous feeding provoked also an increase of the volumetric productivities. However, no attention was paid again to the F/M ratio which in this case was of 2.7 Cmol VFA/(Cmol X · cycle). In this sense, Serafim et al. (2004) have observed in an indirect way that the yield of produced polymer per consumed substrate increases with the acetate concentration. Considering that the amount of biomass did not increase in the same proportion the F/M becomes higher up to a minimum value which allows for the obtaining of the maximum storage capacity (Figure 5.2).

From the evaluated data (Figure 5.2), an inhibitory effect due to a high substrate concentration seemed to appear to F/M values higher than 7 Cmol VFA/(Cmol X · cycle), which corresponded to a value of approximately 350 Cmmol VFA/g VSS.

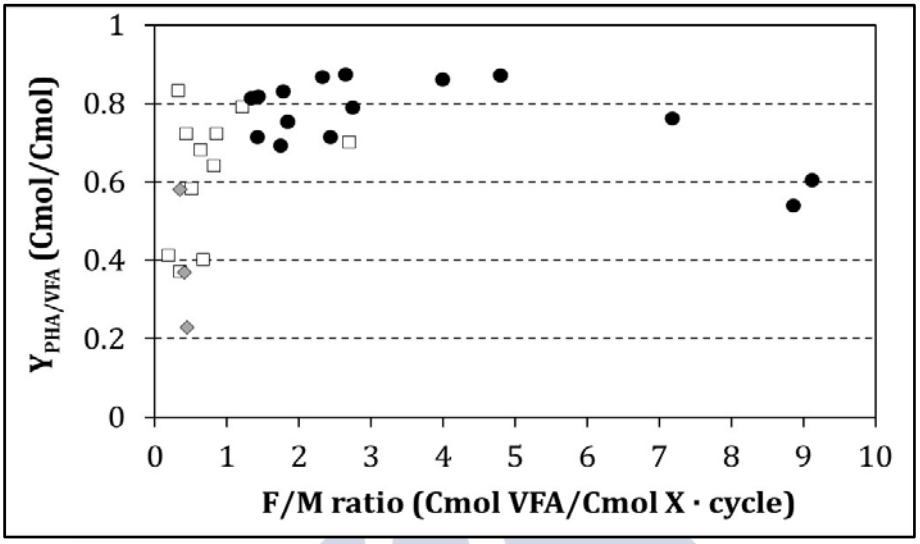

Figure 5.2. Relationship between the PHA yields of per substrate consumed and the ratio F/M Cmol VFA/(Cmol X · cycle) for the accumulation experiments. Data from Serafim et al. (2004) (□), Lemos et al. (2006) (◇), and this study (●).

5.4.6 Microbiology of the selected MMC

The MMC from the enrichment SBR was characterized by molecular techniques and the approximate percentage of abundance of the characterized populations was estimated. The bacterial enrichment was mainly composed by *Azoarcus* (~50%), which accounted at least for half of the identified genera (Figure 5.3.A). The other main genera existing in the microbial community were *Comamonas* (~30%) and *Thauera* (~20%) (Figure 5.3.B and Figure 5.3.C, respectively). No positive results were detected for *Gammaproteobacteria* class or *Paracoccus* genus.

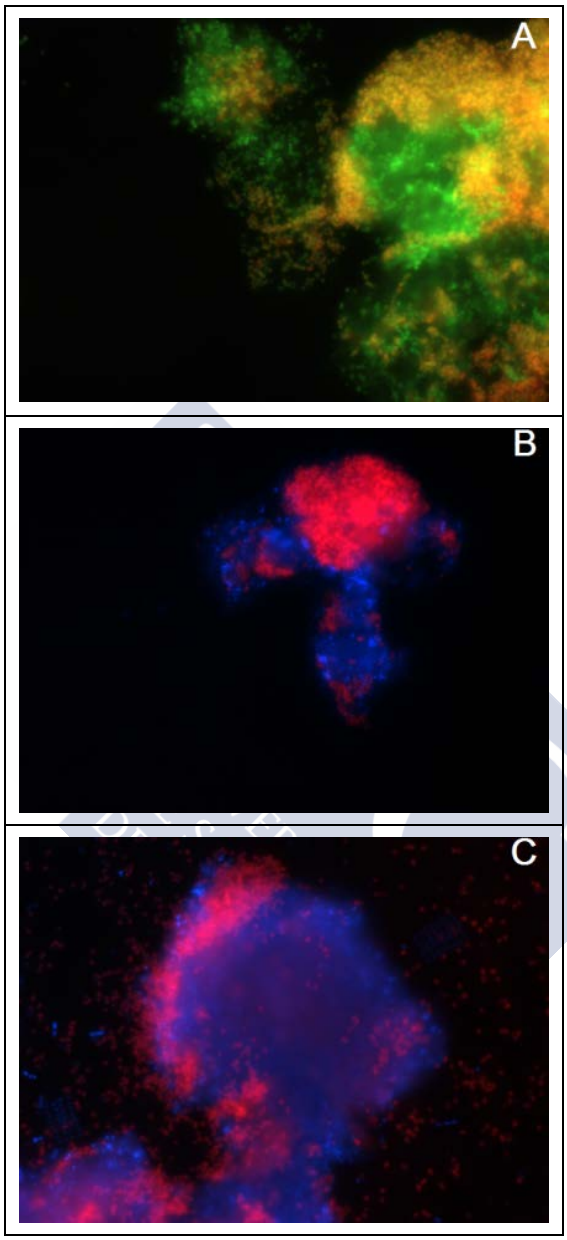

Figure 5.3. FISH images for the enriched MMC characterization. (A) Specific probe for *Azoarcus* genus in orange and $EUB338_{mix}$ for other Bacteria in green. (B) Specific probe for *Comamonas* genus in red and all DNA with DAPI in blue. (C) Specific probe for *Thauera* genus in red and all DNA with DAPI in blue.

It is important to characterise the communities present in the enriched MMC as few authors have previously reported the link of some common genera present in the PHA-accumulating systems with the observed substrate uptake preferences (Albuquerque et al., 2013) and also with the accumulated PHA content and composition (Carvalho et al., 2014). For example, Albuquerque et al. (2013) enriched a microbial community with a mixture of VFAs from clarified fermented molasses (63:11:24:2 Cmol% of HAc:HPr:HBu:HVa). These authors established that *Paracoccus* genus had a moderate substrate preference for HAc and HVa, like *Azoarcus* genus, and high preference for HPr and HBu. However, these authors observed that *Azoarcus* genus had high substrate preference just for HPr while HBu was practically not consumed. *Thauera* genus showed preference for HPr but also experienced a moderate preference for HBu and HVa. In the selected MMC from this study, no positive results for *Paracoccus* genus was detected as this population is barely present in this type of systems (Albuquerque et al., 2013; Lemos et al., 2008).

In the present study, *Azoarcus* and *Thauera* genera accounted for the majority of the MMC population (approximately 70%). These results are in accordance with the experiments performed by Carvalho et al. (2014), who established a correlation between these two genera and the PHA content. They determined an overall yield of about 0.7 CmolPHA/CmolVFA while the values obtained in the present study were in the range of 0.683-0.838 CmolPHA/CmolVFA. Carvalho et al. (2014) also reported that *Thauera* and *Azoarcus* genera were related to high accumulated HV contents. In the present case, the predominance of these two genera is logical as it can be explained by the fact that they have a well-known preference for HPr, which nearly contributed to the 30% of the COD of the VFA mixture used for the MMC enrichment.

5.5 CONCLUSIONS

An appropriate enrichment of a MMC can lead to a versatile production of biopolymers with compositions ranging from HB:HV of 94:2 to 24:76 (wt% of PHA). This variable HB and HV content is achieved by two requirements: (1) the application of a selective pressure in the form of a variable composition of VFAs in the fed substrate for achieving an enriched MMC able to uptake diverse types of substrates and obtain a MMC, and (2) the use of different substrate compositions during the accumulation different from those of the enrichment so as to obtain different biopolymer compositions. These two conditions lead to the main achievements of this study:

- Maximum accumulated PHA in the experiments performed with different VFAs used as substrate for a MMC remains almost constant (39.0 - 48.4 wt%).
- A wide HV content (2-76 wt% of PHA) was obtained in the accumulation experiments when different VFAs were used as substrate. The substrate used for the enrichment is preferred by the MMC providing the highest substrate uptake and biopolymer production rates, although other VFAs are also satisfactorily accumulated depending on their concentration.
- The fact that the MMC was enriched in a VFA mixture provided with high biopolymer yields (0.683 - 0.838 Cmol PHA/Cmol VFA), biopolymer production rates (0.184 - 0.338 Cmol PHA/(Cmol X · h)) and substrate uptake rates (0.275 - 0.439 Cmol VFA/(Cmol X · h)) for all the substrates tested in the accumulation assays. Nonetheless, there were small differences among all the values that can be attributed to the different pathways depending on the substrate.
- The F/M ratio (Cmol VFA/(Cmol X · cycle)) has been identified as a parameter affecting the PHA accumulation yield. An evaluation of the data obtained in the present work together with those from the literature indicated that the maximum yield of approximately 0.8 Cmol PHA/Cmol VFA is obtained for F/M ratios between approximately 1 and 7 Cmol VFA/(Cmol X · cycle). Lower values than 1 Cmol VFA/(Cmol X · cycle) indicate carbon source limitation and higher values than 7 Cmol VFA/(Cmol X · cycle) the appearance of substrate inhibition.

To sum up it can be stated that the use of different fermented substrates leads to different biopolymer compositions while maintaining similar production yields. This is especially useful when working with organic wastewaters that may have variable composition, like vegetable or fish canning wastewaters due to seasonality, and/or industries treating different products and producing also different waste streams.

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Chapter 6

TRANSIENT CONCENTRATIONS OF NACL AFFECT PHA ACCUMULATION IN MIXED MICROBIAL CULTURES

SUMMARY

The present study explores the feasibility of the accumulation of polyhydroxyalkanoates (PHAs) under the presence of transient concentrations of added sodium chloride, by means of a mixed microbial culture (MMC). This culture was enriched on a mixture of volatile fatty acids (VFAs) containing 0.8 g Na^+/L as NaOH. This MMC presented a maximum PHA accumulation capacity of 53 wt% with 27 Cmol% HV.

Accumulation experiments performed with added NaCl at concentrations of 7, 13 and 20 g/L shown that this salt provoked a decrease of the biomass PHA production rate, with an IC_{50} value close to 7 g NaCl/L. The accumulated PHA was lower than the corresponding value of the assay without the addition of salt. Furthermore, the composition of the biopolymer, in terms of HB:HV ratio, changed from a of 2.71 to 6.37 Cmol/Cmol, which means a HV decrease between 27 and 14 Cmol%. Summarizing, the PHA accumulation by a MMC non-adapted to saline conditions affected the polymer composition and lead to lower production yields and rates than in absence of added NaCl.

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6.1 INTRODUCTION

Organic matter recovery from wastewater intends to comply with new regulations from the European Union (Directive 2008/98/EC) which aim at considering wastes as resources to obtain value added products, such as polyhydroxyalkanoates (PHAs).

PHAs are polymers of biological origin, well-known due to their utility for several applications such as raw materials for the production of packaging, medical or building materials. Their advantages rely on being easily biodegradable and the fact that they can be produced from renewable sources (Chen, 2010) or even applied as biofuels (Zhang et al., 2009).

Up to date, most of the research works on PHA have been performed with pure cultures or genetically modified organisms and using valuable industrial by-products as substrate. However, at the moment, the interest is moving to the application of microbial mixed cultures (MMCs), using waste streams as substrate characterized by their large organic matter content. In this latter case, PHA can be produced by MMCs selected by the exploitation of the ecological role of PHA as a microbial storage material (van Loosdrecht et al., 1997), when dynamic operational conditions are applied to the system. A selective pressure is imposed, favouring the selection and growth of different microbial cultures able to have clusters of internal carbon as reservoirs (Beun et al., 2002). The enhanced capacity of the microbial communities to store PHA under these transitory conditions was confirmed by several authors using as carbon source synthetic substrates or different types of wastewater (Albuquerque et al., 2007; Bengtsson et al., 2008; Dionisi et al., 2005; Jiang et al., 2011b; Johnson et al., 2010; Moralejo-Gárate et al., 2011; Reis et al., 2003).

When waste streams are used as substrate for PHA production, certain aspects have to be taken into consideration like the presence of salts, the possible existence of inhibitory compounds, seasonal composition variability, etc. Wastewater streams produced in different food industries are suitable as organic matter source for PHA production (Nikodinovic-Runic et al., 2013), although in some cases they may contain significant amounts of NaCl or even transient concentrations of this salt (Mendez et al., 1995). This is the case of the fish-canning wastewaters, which are characterized by a suitable composition for PHA production in terms of organic matter content. This type of wastewater does not have variations just in terms of organic matter

content but also with respect to the NaCl concentration, due to the seasonality of the product, the location of the plant, the type of seafood processed and/or the processing procedure. For example, on a seaside factory, the octopus boiling wastewater contains 1.33 g NaCl/g COD while the mussel boiling wastewater has 2.24 g NaCl/g COD, and the fish flour line wastewater has 0.23 g NaCl/g COD (Soto et al., 1990). All these products can be processed in the same plant at different moments of the year.

The transitory presence of sodium chloride affects the activity of many different microbial communities in biological wastewater treatment systems (Uygur and Kargı, 2004; Wang et al., 2005). In this sense, the evaluation of the effects of large concentrations of salts has already been the aim of several research works performed using pure cultures. In the specific case of sodium chloride effects, contradictory results have been reported. For example, a progressive reduction of the accumulated PHB, from glycerol as carbon source, by *Paracoccus denitrificans* and *Cupriavidus necator* (strain JMP 134) was observed at concentrations above 5 g NaCl/L, reaching an inhibition percentage of 80% at 20 g NaCl/L (Mothes et al., 2007). On the other hand, Passanha et al. (2014) identified a positive effect of the addition of sodium chloride on the PHA storage of *Cupriavidus necator* when using acetic acid as substrate. They found that the maximum PHA content was achieved at 9 g NaCl/L while biomass was inhibited at above 15 g NaCl/L. This seems to indicate that the sensitivity to NaCl depends on the type of microorganism involved or on the carbon source used as substrate (Passanha et al., 2014).Regarding the effect of sodium ion on PHA accumulating organisms, Mozumder et al. (2015) observed a detrimental effect over the PHA production of *Cupriavidus necator*. PHB production was totally stopped at 10.5 g Na^+/L and no biomass growth was observed at 8.9 g Na^+/L (Mozumder et al., 2015). Available information shows inconclusive results for the consequences of the presence of NaCl on pure cultures while, to the best knowledge of the authors, these effects have not yet been evaluated on mixed cultures.

6.2 OBJECTIVES

The main objective of the present study was to investigate the influence of transient concentrations of sodium chloride, up to 20 g NaCl/L, over the PHA accumulation capacity of a mixed culture. Estimated kinetic and stoichiometric parameters from obtained PHA-accumulation experiments were used for the evaluation of the culture response.

6.3 MATERIALS AND METHODS

6.3.1 Experimental set-up

Two bench-scale reactors were used. The first one was used for the selection of the MMC (Enrichment reactor) and the second one for the maximization of biopolymer inside the cells (Accumulation Reactor).

Further information about the detailed performance of the enrichment reactor together with its corresponding kinetic and stoichiometric values can be seen on Chapter 5 "Versatile Production of PHAs with Mixed Cultures and Different Substrates".

The accumulation reactor was an aerobic fed-batch reactor that operated at 30 °C, with a useful volume up to 5 L. This reactor was inoculated with the biomass contained in the effluent withdrawn from the enrichment reactor. The dissolved oxygen (DO) concentration was measured online and used to control the addition of the VFAs mixture throughout the length of the experiment. pH was measured but not controlled.

6.3.2 Operational conditions

6.3.2.1 Enrichment Reactor

Further information about the detailed performance of this reactor can be found on Chapter 5 "Versatile Production of PHAs with Mixed Cultures and Different Substrates".

6.3.2.2 Accumulation Reactor

Assays were performed using as feeding the same carbon source composition at different concentrations of sodium chloride.

The tested concentrations were 0, 7, 13 and 20 g NaCl/L. These concentrations were maintained constant throughout each experiment. The experiment performed without the addition of NaCl was considered the reference one because it corresponded to the enrichment conditions of the biomass. Feeding was added in pulses of 26.6 Cmmol every time the DO concentration experienced a sharp increase. The reactor was operated under excess of organic matter in order to avoid limitations for biopolymer storage inside the cells.

Data collected from all these experiments was used to calculate the mass balances and kinetic parameters.

6.3.3 Analytical Methods

Liquid samples taken from the reactors were filtered with a mixed cellulose ester filter of 0.45 μm pore size (Advantec, Japan). VFA concentrations were measured following a the method described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005) by gas chromatography (GC) (Hewlett Packard 5890A, USA). The concentrations of total ammonium (NH_4^+) were determined spectrophotometrically (Bower and Holm-Hansen, 1980). pH was measured with an electrode (52-03, Crison Instruments, USA). The temperature was determined with a digital thermometer. The DO concentration was measured and acquired with an oxygen pocket meter (Hach-Lange, USA).

The concentrations of Total (TSS) and Volatile Suspended Solids (VSS) were determined according to the methods described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). PHA was extracted and analysed by a chromatographic method (Smolders et al., 1994).

Respirometric batch assays (López-Fiuza et al., 2002) were performed in a biological oxygen monitoring device model BOM5300 (YSI Inc., USA). The effects of four different concentrations of sodium chloride (5, 9, 13 and 18 g NaCl/L) over the organic matter oxidizing maximum specific activity (MSA) of the MMC were evaluated. Experiments were performed at 30 °C in hermetically closed vials of 10 mL with concentrations about 1 g VSS/L. The liquid media inside the vessels was bubbled with air for 15 min to achieve oxygen saturation conditions (7.6 mg O_2/L at 30 °C).

All these methods are described in detail in Chapter 2 “Materials and Methods”.

6.3.4 Calculations

Calculations related to kinetic and stoichiometric values for PHA production and carbon balances, the amount of PHA, active biomass, CO_2, rates and also the yields for each produced compound were determined in each accumulation assay. All these calculations are described in detail in Chapter 2 “Materials and Methods”.

6.4 RESULTS AND DISCUSSION

A set of accumulation experiments were carried out to study the effects of the presence of added sodium chloride over the biomass activity, the PHA-accumulation and the composition of the stored biopolymer. Two types of assays were performed so as to evaluate the effect of NaCl concentration: (1) respirometric batch experiments in order to determine the effect over the oxidizing activity and (2) accumulation cycle measurements to study the influence over the PHA-composition and the storage capacity of the MMC.

The enrichment reactor and its operation has been previously detailed. on Chapter 5 "Versatile Production of PHAs with Mixed Cultures and Different Substrates".

6.4.1 NaCl effects over the oxidizing maximum specific activity

The maximum specific oxidizing activity (MSA) of the biomass was estimated from the respirometric batch experiments using a control media without the addition of sodium chloride (0 g NaCl/L). The maximum obtained value was of 12.5 [g O_2/(g VSS · d)] (Table 6.1).

The inhibitory percentages estimated from experiments performed at concentrations of 5, 9, 13 and 18 g NaCl/L revealed a great influence of the presence of NaCl over the MSA of the biomass (Table 6.1). Even small concentrations of sodium chloride provoked a negative effect over the non-adapted biomass. The IC_{50} value corresponded to a concentration of approximately 5 g NaCl/L. Higher sodium chloride concentrations caused a more severe effect over the respiration activity which continued diminishing with the increase of the NaCl concentration. A value of 78.5% was obtained for the highest tested concentration of 18 g NaCl/L and 85.5% at 13 g NaCl/L. These results indicated that the activity was almost negligible at values higher than 13 g NaCl/L. The slight difference between the two highest inhibition values can be attributed to errors in the measurements.

These results are in accordance with those obtained by other authors who performed experiments evaluating the oxygen uptake rate (OUR) of activated sludge under the presence of NaCl. They detected the inhibition of the respiration activity by the presence of sodium chloride in the liquid media and determined an IC_{50} value of about 22 g NaCl/L (Pernetti et al., 2003). In the present case, this value is much lower

presumably due to the fact that the biomass is not used to perform the respiration activity as the main process but the PHA accumulation one and this makes it more sensitive to this salt. For this reason, to evaluate the effect of this salt on the PHA accumulation process is required.

Table 6.1. Effect of the NaCl concentration over the organic matter oxidizing Specific Activity (SA) of the PHA-producing community.

NaCl (g/L)	Inhibition (%)	SA (g O_2/g VSS d)
0	0	12.48
5	50.5	6.17
9	58.7	5.14
13	85.5	1.81
18	78.5	2.68

6.4.2 NaCl effects over PHA accumulation

The effects of sodium chloride over the accumulation capacity and obtained biopolymer composition of the MMC non-adapted to the presence of salt were evaluated by cycle measurements performed with different amounts of added sodium chloride of 7, 13 and 20 g NaCl/L.

The accumulation experiments were run for 24 hours (Figure 3). In the four experiments, HB production presented an upward trend with different slopes corresponding to different production rates (Figure 3). The same observation is applicable for HV content which had an uprising trend, except for the experiment performed at 20 g NaCl/L, where the percentage remained almost constant meaning that no significant HV was synthesised. From these obtained results, inhibitory effects over the PHA production rate of 50% were estimated to correspond to a concentration about 7 g NaCl/L. This value is similar to the IC_{50}value for the VFA oxidation activity (5 g NaCl/L), previously determined from respirometric assays.

The cycle performed at 13 g NaCl/L showed degradation of the produced polymer after reaching the maximum value of accumulated PHA. The same behaviour was observed for the assay at 20 g NaCl/L and, for this reason, the corresponding

assay in Figure 6.1 is represented till the maximum accumulated PHA amount was reached at 500 minutes from the beginning of the batch assay. Degradation of the stored biopolymer under the presence of NaCl has been previously observed in studies using pure cultures of *Cupriavidus necator*, where it occurred at all the tested concentrations (3.5 to 15 g NaCl/L) and was faster for the accumulation assay at 9 g NaCl/L (Passanha et al., 2014). Furthermore, PHA degradation was explained as a reaction to overcome the stress produced by the presence of NaCl but no explanation for this was provided (Passanha et al., 2014). In the present case, it was observed that the pH decreased to values lower than 6.3 when the VFA uptake capacity was exceeded and, for this reason, only the accumulated PHA could be used as substrate.

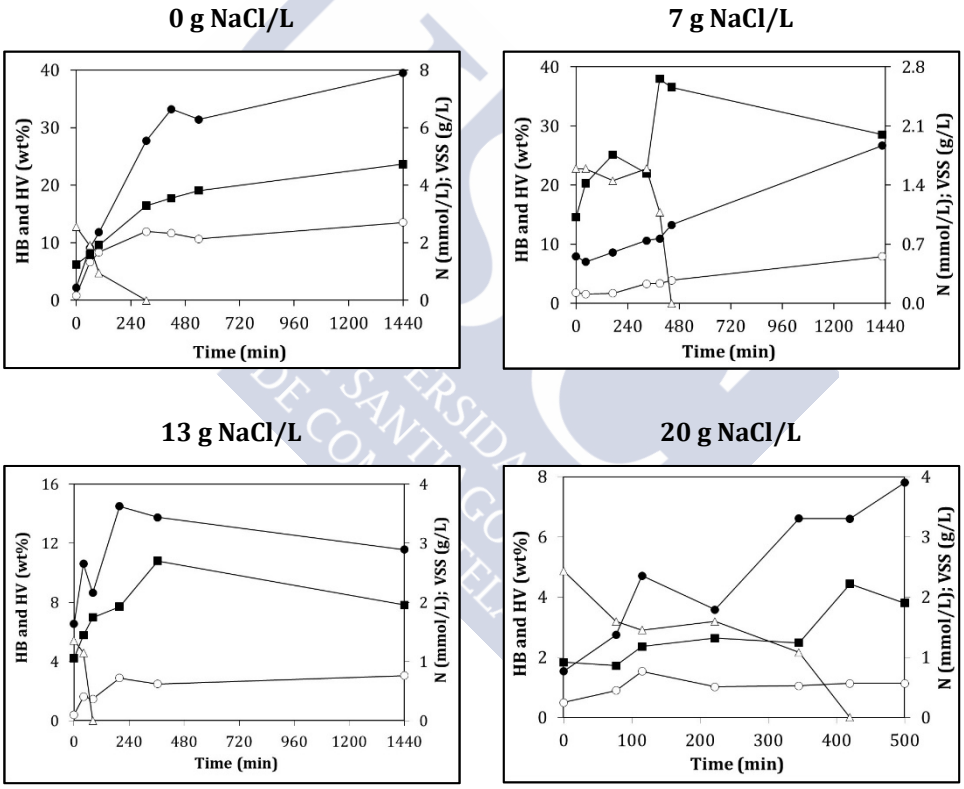

Figure 6.1. Evolution of measured compounds in the accumulation assays performed at different sodium concentrations: 0, 7, 13 and 20 g NaCl/L. NH4+-N (Nmmol/L) (△); VSS (g/L) (■) and percentage of the stored compounds (wt%) as HB (●) and HV (○).

The maximum PHA accumulated also depended directly on the NaCl concentration. PHA in the biomass in dry weight was of 53.0% for the experiment without the addition of sodium chloride while values of 34.6%, 17.4% and 8.9% were obtained for the experiments performed at 7, 13 and 20 g NaCl/L respectively (Figure 6.2-A).

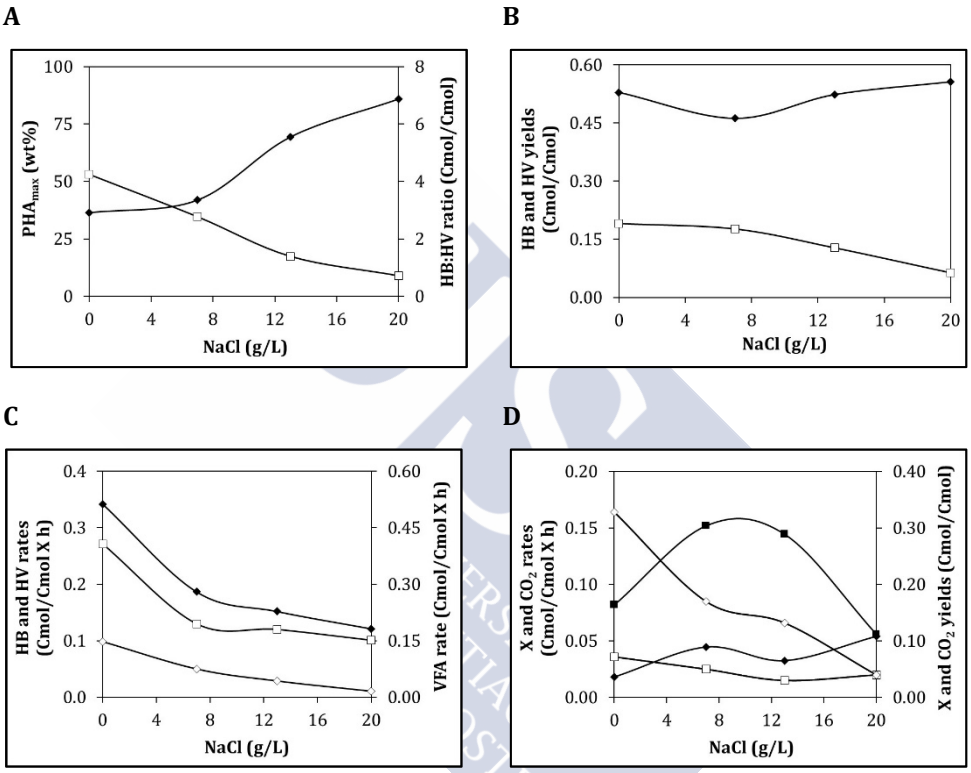

Figure 6.2. Comparison of the measured kinetic and stoichiometric parametersfor the accumulation assays performed under different sodium concentrations: 0, 7, 13 and 20 g NaCl/L. (A) Maximum PHA content (%wt) (□) and HB:HV ratio (Cmol/Cmol) (♦). (B) HV yield (□) and HB yield (♦) (Cmol/Cmol). (C) VFA specific consumption rate (Cmol/Cmol X · h) (♦) HB specific production rate (Cmol/Cmol X · h) (□) and HV specific production rate (Cmol/Cmol X · h) (◊). (D) Biomass specific production rate (Cmol/Cmol X · h) (□), CO_2 specific production rate (Cmol/Cmol X · h) (◊), Biomass yield (Cmol/Cmol) (♦) and CO2 yield (Cmol/Cmol) (■).

The monitoring of the evolution of the monomer synthesis throughout the experiments indicated that the PHA formation rate diminished with the increase of the tested NaCl concentrations (Figure 6.2 - C). The HB production rate for the experiment without NaCl addition was of 0.271Cmol HB/(Cmol X · h) and this value was of 0.130, 0.120 and 0.101 Cmol HB/(Cmol X · h) when sodium chloride was added and its concentration reached values of 7, 13 and 20 g NaCl/L, respectively (Figure 6.2 - C). HV production rates were significantly lower than HB ones with values of 0.098 for the control assay while values of 0.046, 0.029 and 0.011 Cmol HV/(Cmol X · h) were reached for concentrations of 7, 13 and 20 g NaCl/L, respectively.

The measured yields showed two different behaviours: HB yields were not affected by the presence of sodium while HV yields were strongly affected by the effect of the inhibitor (Figure 6.2 - B). On one hand, HB yield had a control value of 0.529 Cmol HB/Cmol VFA and this was nearly the same as the values obtained for the concentrations of 7, 13 and 20 g NaCl/L which were of 0.462, 0.523 and 0.556 Cmol HB/Cmol VFA, respectively. On the other hand, HV yield was noticeably influenced by the presence of sodium chloride. It was of 0.191 Cmol HV/Cmol VFA when no inhibitor was added and values of 0.164, 0.128 and 0.063Cmol HV/Cmol VFA were obtained at concentrations of 7, 13 and 20 g NaCl/L, respectively.

Not only the amount of PHA inside the cells and the production rates were affected by NaCl concentrations but also the composition of the biopolymer was influenced. The HB:HV ratios corresponding to the samples of maximal PHA accumulation were of 2.71, 3.13, 4.67 and 6.37 (Cmol/Cmol) for the experiments at 0, 7, 13 and 20 g NaCl/L respectively (Figure 6.2 - A). These values corresponded to a content of 27, 24, 18 and 14 Cmol% HV, respectively. This increasing evolution of the ratio was related to the decrease of HV in the biopolymer at higher sodium concentrations. Under inhibitory conditions, propionic acid (precursor for HV synthesis) seemed to be more likely used for cell maintenance rather than for biopolymer production in comparison to the other acids.

To explain this hypothesis, it must be indicated that butyrate and valerate acids are not expected to be significantly used for cell maintenance as Pardelha et al. (2014) stated. Acetate and propionate are known to be the most probably eligible VFAs for maintenance because these are activated directly to acetyl-CoA and propionyl-CoA, respectively. This is not the case of butyrate and valerate, which

require the consumption of one more ATP mol to be converted to acetyl-CoA and propionyl-CoA through the β-oxidation pathway (Pardelha et al., 2014).

With respect to the observed preferable use of propionate over acetate for maintenance the energy (as ATP) consumption and production due to the metabolic process must be considered. The stoichiometric consumed energy per amount of acid consumed is of 1:1 mol ATP/mol acetate and 0.67:1 mol ATP/mol propionate(Jiang et al., 2011a), with the consequent lower energy required for propionate than for acetate consumption. Simultaneously, 1:1 mol acetyl-CoA/mol acetate and 1:1 mol of propionyl-CoA/mol propionate are produced (Jiang et al., 2011a).

The amount of generated energy from the CoA molecules corresponding to the conversion of 1.5 mol of propionyl-CoA to 1 mol of acetyl-CoA that liberates 1.5 moles of $NADH_2$ (Pardelha et al., 2014; Tamis et al., 2014) that produces ATP through the oxidative phosphorylation cycle. From these data, it is inferred that the transformation of propionyl-CoA, coming from propionate consumption, has higher potential for energy production.

This implies a general energy balance favouring the production of more ATP when using propionate instead of acetate for cell maintenance and leading to lower production of HV when inhibition becomes stronger. This hypothesis can be strengthened with the results shown for the production rates and the yields (Figure 6.2 - B) as there were almost no different results for HB yields but remarkable differences were observed for HV yields as propionic acid is preferable consumed for maintenance. It was also observed that HV production rates were more affected than HB ones under the presence of sodium chloride. HB specific production rates were not significantly different for the values obtained at 7, 13 and 20 g NaCl/L although the HV specific production rate for the same concentrations was nearly half the value without salt addition. This observation supports the idea given before that HV synthesis is lower than in the case without NaCl addition because of the use of propionic acid for maintenance. At 20 g NaCl/L the inhibition was very strong and the HV specific production rate was almost negligible (Figure 6.2 - C), being the HV concentration more or less constant during most of the time of the experiment (Figure 6.1).

Results from this research study indicate that the presence of sodium chloride changed the amount of biopolymer inside the cells and the HB:HV ratio which is an

indicator of the properties of the polymer. This modification of the properties affects the possible further use of these materials.

It is important to remark that this MMC was enriched with a medium containing 0.8 ± 0.07 g Na^+/L. Higher resistance to NaCl sensitivity could be expected if this mixed community was enriched at high sodium chloride concentrations. However, the possible influence of the salinity on the HB:HV ratio and changes in the favoured metabolic pathways should be further researched.

6.5 CONCLUSIONS

The main conclusion obtained from this study is that the presence of added sodium chloride in the feeding media affects the PHA accumulation performance of a MMC non-adapted to saline conditions. These effects are summarized as:

- The amount of stored biopolymer inside the cells decreased when the NaCl concentration increased.
- The HB:HV ratio increased as the concentration of inhibitor also increased. This parameter is a rough indicator of the properties of the produced biopolymer, demonstrating that they can be changed by varying the concentration of an inhibitor like NaCl.
- The stoichiometric and kinetic parameters depended on the salt concentration in such a way that the biopolymer production was lowered severely at higher sodium chloride concentrations. Also the measured HV yields diminished because of the use of the substrate presumably for maintenance and not for accumulation purposes.

All these parameters where studied to evaluate the influence of NaCl transient shocks over the PHA-accumulating biomass. If it is expected to operate with saline conditions, the characteristics of the enriched MMC are expected to be the key factor so as to improve the kinetic parameters.

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Chapter 7
PHA PROPERTIES AND ITS AFFECTATION BY THE PURIFICATION STAGE OR THE PRESENCE OF NaCl

SUMMARY

The mechanical properties of bacterial polyhydroxyalkanoates (PHAs) can be tailored by changing the type of substrate in the accumulation stage. Hence different comonomer distributions can be produced with significant variations in their physico-chemical properties. In the present study, the biopolymer produced by a mixed microbial community enriched in PHA accumulators fed with different substrate composition was characterised. The accumulation experiments were: single acetic (A1) and propionic acids (A2); an equivolumetric mixture of acetic and propionic acids (A3); volatile fatty acids (VFA) mixture (A4); and this VFA mixture and a concentration of 21.6 g NaCl/L in the reactor (B1). The objective was to investigate the impact of different circumstances on biopolymer composition and properties. The obtained copolymers were constituted by units of hydroxybutyrate (HB) and hydroxyvalerate (HV) with HB:HV ratios between 96:4 and 34:66 (wt%).

Thermal properties showed a clear dependence on the HB:HV ratio although no apparent connection with the decomposition temperature was found. Salinity affected both biopolymer composition and properties. The physico-chemical properties of purified and no purified polymer were also determined. Purification was found not to affect the molecular weight, while crystallinity augmented more than 70% and the thermal properties also increased. This study shows that, besides the substrate composition, also salts present in the media and the purification stage affect the properties of the produced biopolymer.

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7.1 INTRODUCTION

Polyhydroxyalkanoates (PHAs) are biodegradable and biobased polyesters which, in terms of application, have thermoplastic properties (Lee, 1996) and are biocompatible (Reddy et al., 2013). These features are interesting for applications such as packaging materials, fine chemicals, hygiene products or medical purposes, among others (Chen, 2010; Soroudi and Jakubowicz, 2013). They are produced naturally by several microorganisms as water-insoluble granules which are stored inside the cells and serve them as energy source (Sudesh and Abe, 2010; Wang et al., 2001). PHA production, using mixed microbial communities (MMCs) together with wastes as substrate, has been focus of attention for the last years due to the associated significant reduction of production costs up to 50% in comparison with current commercial processes using pure cultures (Reis et al., 2003). In this way, wastewaters serve as feedstock for biopolymer production while these streams are treated so as to meet the discharge requirements.

The most well-known PHA is polyhydroxybutyrate (PHB) (Roy and Visakh, 2015). The properties of P3HB are usually comparable to those of polypropylene, although the first is more brittle than the second one (Sudesh et al., 2000) which seem to limit its application. Nevertheless, P3HB presents other important added advantages like its low oxygen and water-vapour permeability (Shen et al., 2009), which are considered, together with its tensile properties, as key factors for food packaging applications (Sudesh et al., 2000). However, the incorporation of different monomeric units in PHA copolymers can improve the biopolymer properties like the impact resistance (Imre and Pukánszky, 2013). As an example, the introduction of HV monomers in the biopolymer chains reduces its crystallinity and the values of the melting and glass transition temperatures (Modi et al., 2011), enhancing the flexibility and processability of the biopolymers (Dias et al., 2006; Shen et al., 2009).

PHA properties can be tailored by controlling the monomer distribution (Laycock et al., 2013; Sudesh et al., 2000). Reported crystallinity for PHB ranges from 30-56% with molecular weights (M_w) in the interval of $3.5x10^5$-$3x10^6$ g/mol while these values differ significantly for PHBV copolymers. Crystallinity ranged from 4 to 34% and M_w from $1.1x10^5$ to $2.9x10^6$ g/ml for different copolymers with HB:HV distributions between 95:5 and 28:72 mol% (Duque et al., 2014; Hilliou et al., 2016; Reis et al., 2003; Serafim et al., 2008). The biopolymer composition also affects the thermal characterization of PHAs in MMCs under aerobic dynamic feeding, in terms

of glass transition temperature (T_g), melting temperature (T_m) and decomposition temperature (T_d). PHBV copolymers improve the melt stability comparison with PHB polymers (Modi et al., 2011) but without reducing the thermal stability of the material (Castro-Mayorga et al., 2016). Reported thermal values for different HB:HV ratios ranged between -29.6 °C and 56 °C for T_g, and between 70.7 °C and 175.3 °C for T_m (Albuquerque et al., 2011; Duque et al., 2014; Hilliou et al., 2016; Laycock et al., 2014; Reis et al., 2003; Serafim et al., 2008). The values for T_d ranged in general between 226.8 °C and 305 °C for copolymers obtained by MMCs. The intervals for the reported values of thermal properties vary significantly, opening the possibility for a wide range of applications.

Although there is some information regarding the physico-chemical properties of the biopolymers produced in MMCs, there is little information about the influence of other parameters over the composition and properties. There are some operational conditions known to affect the biopolymer properties: the feeding regime (pulse or continuous) (Albuquerque et al., 2011); the alternation of different feedings (Arcos-Hernández et al., 2013); or even the purification of the extracted sample (Hilliou et al., 2016). Furthermore, it is known that other variables like the pH of operation (Villano et al., 2010) or the presence of salts (like NaCl) in the medium affect the monomer distribution (Palmeiro-Sánchez et al., 2016). Though the way these variables affect the properties has not been reported yet.

Generally, the variability of the PHA properties produced by MMCs is mainly attributed to the substrate composition. However, the effects are not always obvious when variable feeding compositions are supplied. This involves the necessity of researching for each substrate composition the characteristics of the resulting produced biopolymer but also the influence of other parameters like the presence of salts or the composition of the enrichment medium.

7.2 OBJECTIVES

In the present study, three objectives were pursued:

- To assess the influence over the biopolymer properties of the variability of the substrate.
- To study the influence over the biopolymer properties of the presence of NaCl in the medium.

- To give more light to the influence of the purity over the sample properties.

So as to achieve these three objectives, different substrates were tested for the accumulation assays; one of the substrates was tried with and without NaCl presence to assess the salinity influence during the accumulation; and two different extraction methods were tested.

7.3 MATERIALS AND METHODS

7.3.1 Experimental set-up

The biopolymers were produced in a two-stage process: (1) the culture selection stage, where the PHA-producing microbial community is developed fed with a mixture of volatile fatty acids (VFAs) under aerobic dynamic feeding conditions (feast-famine regime); and (2) the biopolymer production stage, where a fed-batch accumulation reactor, operated under excess of organic matter, was used for maximizing the PHA content inside the cells.

The enrichment reactor and its operation has been previously detailed on Chapter 5 "Versatile Production of PHAs with Mixed Cultures and Different Substrates".

The accumulation reactor was an aerobic fed-batch reactor, with a volume up to 5 L. This reactor was inoculated with the biomass contained in the effluent withdrawn from the enrichment reactor and it also operated at a temperature of 30 °C. pH was measured but not controlled. The dissolved oxygen (DO) concentration was measured online and used to control the addition of the VFAs mixture throughout the length of the experiment.

7.3.2 Operational Parameters

Operational parameters are described for the accumulation assays since the enrichment conditions have been previously defined on Chapter 5 "Versatile Production of PHAs with Mixed Cultures and Different Substrates".

The DO was pumped through a diffuser and its concentration fluctuated between a minimum of 0.2 and a maximum of 6.6 mg O_2/L in the assays A1 to A4 while assay B1 fluctuated from 7.1 and 7.8 mg O_2/L. A new VFA pulse was added each time the concentration of DO rose up, indicating the depletion of the substrate

added in the previous pulse. The concentration of the pulses was between 20 and 30 Cmmol/$L_{reactor,}$ apart from the assay with NaCl, which had an initial concentration of 50 to a final concentration of 25 Cmmol/$L_{reactor}$. The reactor was jacketed and the temperature controlled at 30 °C by a thermostatic bath (Techne Inc., USA). The pH was not controlled but it was measured online.

These fed-batch assays were performed to determine the maximum accumulation capacity of the enriched microbial community. Ammonia concentration was limited during all accumulation experiments in order to prevent the biomass growth. Throughout the accumulation assays, samples from the liquid and solid phases were periodically collected for analysis.

A set of assays was performed by feeding the enriched MMC with synthetic media with different VFA compositions:

(A1) 100% Acetic acid

(A2) 100% Propionic acid

(A3) 50/50 vol% of acetic and propionic acids

(A4) VFA mixture used in the enrichment reactor

(B1) VFA mixture used in the enrichment reactor and at concentration of 21.6 g NaCl/L in the reactor

The biopolymers produced in these experiments were characterised in terms of PHA content, monomer distribution, thermal properties, and molecular weight.

7.3.3 Analytical Methods

Liquid samples taken from the reactors were filtered with a mixed cellulose ester filter of 0.45 μm pore size (Advantec, Japan). VFA concentrations were measured following a the method described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005) by gas chromatography (GC) (Hewlett Packard 5890A, USA). The concentrations of total ammonium (NH_4^+) were determined spectrophotometrically (Bower and Holm-Hansen, 1980). pH was measured with an electrode (52-03, Crison Instruments, USA). The temperature was determined with a digital thermometer. The DO

concentration was measured and acquired with an oxygen pocket meter (Hach-Lange, USA).

The concentrations of Total (TSS) and Volatile Suspended Solids (VSS) were determined according to the methods described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). PHA was extracted and analysed by a chromatographic method (Smolders et al., 1994).

The PHAs were recovered with chloroform and purified by selective precipitation with ethanol. The average molecular weight, M_w, number average molecular weight, M_n, and polydispersity, M_w/M_n were determined by size exclusion chromatography (SEC) (Waters, USA). Differential scanning calorimetry (DSC) was used in order determine the thermal properties of the obtained biopolymers in terms of T_g, T_m, and melting enthalpy (ΔH_m). The film extracted with chloroform was digested again using the modified acidic methanol method (Serafim et al., 2004) and analysed by GC-MS. Afterwards, the same film sample was purified using the chloroform-ethanol method (Koller et al., 2013; Ramsay et al., 1994). The purified biopolymer was recovered as powder and it was digested by the modified acidic methanol method so as to prepare the sample for GC-MS analysis.

All these methods are described in detail in Chapter 2 "Materials and Methods".

7.3.4 Calculations

Calculations related to kinetic and stoichiometric values for PHA production and carbon balances, the amount of PHA, active biomass, CO_2, rates and also the yields for each produced compound were determined in each enrichment and/or accumulation assay. All these calculations are described in detail in Chapter 2 "Materials and Methods".

7.4 RESULTS AND DISCUSSION

The biopolymer composition and, hence, its properties are known to be affected by the substrate composition supplied during the accumulation stage in MMCs, as reported previously by some authors (Duque et al., 2014; Lemos et al., 2006). In this section, three influencing variables are studied and discussed: the influence of the feeding composition during the accumulation experiments, in terms of VFAs

mixtures and the NaCl content on the PHA properties and of the purity of the biopolymer on its structural and thermal properties.

The description of the operation of the enrichment reactor is described on Chapter 5 "Versatile Production of PHAs with Mixed Cultures and Different Substrates".

7.4.1 Influence of the substrate composition

Four batch assays (A1-A4) were performed applying different VFA composition for the determination of the physico-chemical properties of the produced biopolymers with the same selected MMC. When acetate was the sole substrate (A1) a maximum PHA amount of 53.8wt% was achieved with a HB:HV ratio of 96:4 (Table 2). In this case HB was mainly produced although a small variation of HV from 0.6 to 1.3 wt% was detected, corresponding to a HV accumulation rate of 0.003 $Cmol_{HV}/(Cmol_X \cdot h)$. This fact can be explained by the fact that part of the acetyl-coA produced from the acetate degradation can enter the pathway which leads to the formation of HV (Lemos et al., 2006; Pardelha et al., 2012). On the other hand, HV was mainly produced when propionate was used as carbon source (A2), in spite of the significant part of propionyl-CoA forming acetyl-coA, which lead to the production of HB (Jiang et al., 2011; Lemos et al., 2006; Pardelha et al., 2014) with a HB:HV ratio of 34:66 and a maximum PHA content of 46.3wt%. The samples A3 and A4 lead to an accumulation percentage of 54.5wt% and 51.3wt% PHA, respectively, with the corresponding HB:HV ratios of 44:56 and 62:38. The main issue of this system consisted on demonstrating the feasibility of tailoring the biopolymers by changing the feeding media composition and concentrations used for the accumulation assays maintaining almost the same PHA recovery (*article submitted*). In order to give more information about this versatile production, the physico-chemical properties were also determined.

Measured T_g values ranged from -21.0 °C to -6.6 °C while T_m ranged from 91.7 °C to 161.4 °C (Table 2). The lower T_g and T_m values were obtained for the biopolymers with higher HV content, as a consequence of the presence of greater amounts of propionic acid in the feeding. The same response of lower T_g and T_m at higher HV contents was reported by other authors (Albuquerque et al., 2011; Arcos-Hernández et al., 2013; Duque et al., 2014). The variation of the T_g values with the HB%, alike the T_m ones, can be observed on Figure 1.A.

In all cases, only one T_g was observed for each sample, indicating that homogeneous copolymers were formed or that all the substances present in the melt may be miscible (Arcos-Hernández et al., 2013). On the other hand, in some of the analysed cases, two values for T_m were obtained (Table 2) due to the appearance of two differentiated melting peaks in the thermogram. These multiple melting peaks could be due to the compositional heterogeneity of the biopolymer. However, some authors reported that these multiple DSC peaks could be also due to other variables like melting, re-crystallization, and re-melting during heating, different molecular weight species, orientation effects among several others (Arcos-Hernández et al., 2013; Liu and Petermann, 2001). Another feasible explanation is that both melting point represent firstly the melting of HV blocks and secondly the HB ones, as this blocky structure was a consequence of the pulse-wise feeding in the fed-batch reactors (Hilliou et al., 2016).

The T_d for each sample A1 to A4 was in the range between 226.3 °C and 250.0 °C (Table 2). Values of commercial PHBV copolymers were of 266.7 °C for P3HB and 273.3 °C for P3HB3HV with 10 mol% of HV (Laycock et al., 2013). No clear dependence was found with the composition of the biopolymer, in terms of HB% (Figure 1.B), as it was found previously by other authors (Albuquerque et al., 2011; Bengtsson et al., 2010). As T_d values were above 200°C, no degradation below this value was expected. It is also relevant for application purposes that T_d values of the obtained biopolymers are much higher than their respective T_m ones because this fact allows for the use of different types of thermal processing for these materials.

Higher HB contents resulted in higher crystallinity (Figure 1.B). The exception to this correlation was found on the assay fed with propionic (A2) which had a crystallinity of 10.5%.

The molecular weight distributions (PDI 1.5-1.6) were narrow and independent of the HB% although the M_w values correlated to HB% values (Figure 1.C). The M_w measures in the samples A1 to A4 ranged between 8.7 x 10^4 and 1.5 x 10^5 g/mol. Values in the same order of magnitude have been reported for other mixed cultures although they presented higher values (g/mol): 4.4 - 5.9 x 10^5 (Arcos-Hernández et al., 2013); 3.5 - 9.0 x 10^5 (Bengtsson et al., 2010) and 2.2 - 4.4 x 10^5 (Duque et al., 2014). These differences could be due to several factors that affect the M_w values like substrate type used for the enrichment, its concentration, temperature, operational pH or even the differences between the involved microbial communities.

Table 7.1. Results for the chemical and thermal characterization for the PHA produced in the batch experiments.

Substrate	PHA (%)	HB:HV ratio (HB:HV wt%)	Purity (%)	T_g (°C)	T_m (°C)		ΔH_m (J/g)		T_d (°C)	Crystallinity (%)	M_w (g/mol)	PDI
A1	53.8	96:4	96.6	-6.6	145.4	161.4	5.3	39.6	237.2	24.0	8.7×10^4	1.5
A2	46.3	34:66	89.3	-21.0	91.7	n. d.	55.6	n.d.	240.4	10.5	1.5×10^5	1.6
A3	54.5	44:56	97.0	-12.4	105.0	n. d.	3.4	n.d.	226.3	2.3	1.4×10^5	1.5
A4	51.3	62:38	88.5	-7.7	102.0	158.8	5.7	n.d.	250.0	3.9	1.2×10^5	1.6
B1	8.4	72:28	81.6	-5.2	103.3	166.7	6.5	9.7	256.2	6.7	2.0×10^5	1.3
P-A1*	Pure	95:5	100.0	5.3	165.2	n. d.	72.5	n.d.	270.6	50.1	8.7×10^4	1.3
P-A2*	Pure	43:57	100.0	-11.0	94.8	104.6	31.6	n.d.	269.4	18.4	1.4×10^5	1.8

n.d. – Not detected

*samples further purified with the chloroform-ethanol extraction method

Though there is still the need of well-define the mechanisms controlling M_w (Laycock et al., 2013). Much higher values (2.2 – 3.4 x 10^6 g/mol) were found by other authors (Serafim et al., 2008) although remarkable differences were found in the performance of the assays which could affect the M_w values. The samples were obtained from a SBR with presence of nutrients (Serafim et al., 2008) while in the present study the samples for the analysis of the properties were obtained in a fed-batch reactor without the presence of nitrogen. It is important to remark that other authors observed in pure cultures that the presence of nitrogen source lead to higher M_w values (Shimizu et al., 1993). Another main difference is that the SBR assays were fed with just one substrate pulse (except in 3 cases where 3 pulses were fed) but in any case, not the maximum amount withstood by the system (Serafim et al., 2008). This difference is quite important in terms of properties as these variables influence them (Albuquerque et al., 2011).

7.4.2 Influence of the presence of NaCl

The assay B1 was carried out to investigate the consequences of the presence of sodium chloride in the medium over the biopolymer properties. This was done to mimic the use of fermented feedstocks with variable salinity content, as in the case of fish-canning industries, which have concentrations ranging between 0.5-33.0 g NaCl/L (Soto and Lema, 1998). The substrate in this assay was the same VFA mixture previously used, allowing for the comparison between the biopolymers obtained with and without the presence of NaCl in the reactor (assays B1 and A4).

The maximum accumulation capacity for the MMC was 51.3% and 8.4 %PHA for A4 and B1, respectively. These results indicate that NaCl has a clear inhibitory effect on the accumulation capacity of the MMC. Also the biomass respiration and the kinetic parameters are affected when NaCl is present in the medium, with a clear reduction of activity (IC_{50} = 5 g NaCl/L) as the NaCl concentration increases (Palmeiro-Sánchez et al., 2016). Nevertheless, the main objective of this comparison between assays A4 and B1 was to check the changes in the properties when the same MMC was submitted to the same substrate and the same feeding strategy.

At first sight, the HB:HV ratio changed, 62:38 for A4 and 72:28 for B1, indicating that the physico-chemical properties may have also suffered changes. A possible explanation for this higher HB content in assay B1 is that propionate could be

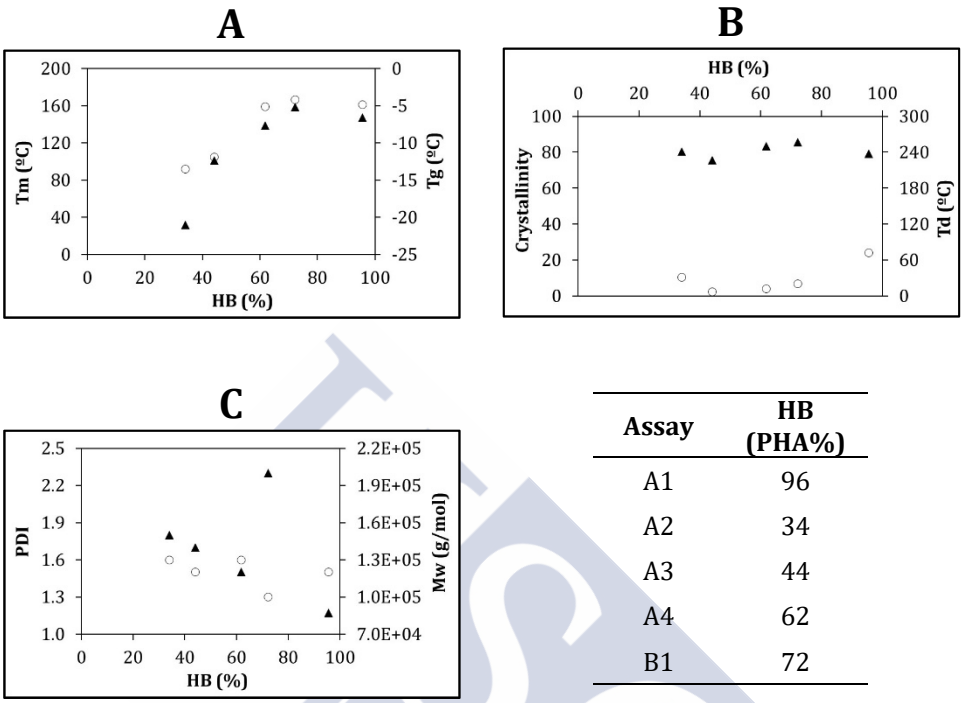
preferred for cell-maintenance under inhibitory conditions rather than acetic acid (Palmeiro-Sánchez et al., 2016).

The value of M_w of 1.2 x 10^5 g/mol was obtained for assay A4 while a value of 2.0 x 10^5 g/mol was achieved for B1, which was the highest value obtained for any of the accumulation assays (Figure 7.1 - C). The M_w is a function of HB% but other factors can affect it significantly (Figure 7.1 - C), leading to higher values. A possible hypothesis is that this increase of the M_w could be due to the fact that the PHAs with lower molecular weight could be degraded faster, resulting in an increase of the M_w, as Shimizu et al. (1993) observed for *Alcaligenes eutrophus* under the presence of nitrogen source (Shimizu et al., 1993). The PDI (1.3) was lower than the values obtained when no sodium chloride was present in the medium (1.6), but all the PDI values (A1-A4 and B1) were in a narrow range (1.3-1.6), indicating a low polydispersity.

Regarding the comparison of the thermal properties, all of the values were dependent on the changes in the HB% rather than other mechanisms due to the presence of NaCl (Figure 7.1).

The decomposition behaviour of the extracted biopolymers changed somewhat, with T_d of 250.0 °C and 256.2 °C for A4 and B1, respectively. Slightly higher crystallinity and enthalpy values were also found when NaCl was present (6.7 %; 9.7 J/g) in comparison with its absence (3.9 %; 5.7 J/g), which is logic due to the higher content in HB, leading to higher crystallinity. The T_m (166.7 °C for B1and 158.8 °C for A4) and T_g (-5.2 °C for B1and -7.7 °C for A4) values were also affected, which is attributable to the higher %HB (Figure 7.1 - A).

As the main conclusion, it can be observed that the presence of NaCl affected the composition in terms of HB:HV and, as a consequence, it affected the thermal properties. However, the M_w was affected at a higher extension, as it is a function of HB% but also of other factors, leading to M_w values higher than expected by just taking into account only its composition.



Assay	HB (PHA%)
A1	96
A2	34
A3	44
A4	62
B1	72

Figure 7.1. Dependence of the physico-chemical properties of the assays A1-A4 and B1 with the HB (PHA%). A. Melting temperature (T_m) (○) and Glass Transition Temperature (T_g) (▲). B. Crystallinity (○) and Decomposition Temperature (T_d) (▲). C. Polydispersity Index (PDI) (○) and Molecular Weight (M_w) (▲).

7.4.3 Influence of the purification stage

Biopolymer extraction can be done by several methods, being halogenated solvents like chloroform one of the most used. Although chloroform also extracts other lipidic materials not related with PHA, the main reason for its wide use is the high extraction yields combined with a high quality of the biopolymer (Ramsay et al., 1994). A highly pure biopolymer may be achieved by submitting the sample to further purification steps by the addition of low-molecular alcohols (i.e., ethanol) (Koller et al., 2013). The impact of sample purity on physico-chemical properties of the biopolymers when using the two different extraction methods was evaluated.

The biopolymers obtained from the accumulations A1 and A2 were extracted using the two aforementioned methods: sole chloroform and the combination of chloroform-ethanol (see Section 2.2 for further information about the methods). The purity of the sample from A1 was 96.6% when using chloroform while this value was 89.3% for assay A2. Results depicted in Table 2 show the behaviour of the different physicochemical properties measured.

The purification stage did not affect the molecular weights, as it was the same for purified and non-purified samples. The M_w, for both A1 and P-A1, was 8.7 x 10^4 g/mol, while the M_w for A2 was 1.5 x 10^5 g/mol and 1.4 x 10^5 g/mol for P-A2.

The most important changes were found in the thermal characteristics. Decomposition temperatures for all non-purified samples were very similar with T_d values between 237.2 °C and 240.4 °C for the non-purified samples fed with HAc and HPr, respectively. Similarly, T_d was, respectively, 270.6 °C and 269.4 °C for the purified samples using HAc and HPr as feedings. Although no dependence was found between T_d and the biopolymer composition, as previously observed (Section 3.1), a significant increase on T_d values when the samples were submitted to the purification step was observed. T_g values were dependent on the biopolymer composition, as indicated in Section 3.1, in such a way that the material with larger HB content presented higher T_g values. T_g was also found to be dependent on the purification degree of the sample as it increased from -6.6 °C to 5.3 °C for HAc sample and from -21.0 °C to -11.0 °C for HPr sample when extracting with sole chloroform and with chloroform-ethanol, respectively. Melting temperatures decreased when the HV contents increased but, again, the purification of the biopolymer influenced this parameter. T_m increased from 161.4 °C to 165.2 °C for HAc sample and from 91.7 °C to 104.6 °C for HPr sample.

Hence, T_g, T_m and T_d increased significantly in purified samples. These findings are consistent with those reported in a recent study by Hilliou et al. (2015), which were focused on the effect of the residues on the characteristics of PHBV produced from MMCs. The decrease of T_d in non-purified samples would be an expected result, based on literature, since residues are reported to negatively affect the thermal stability of biopolymers (Kawalec et al., 2007; Kim et al., 2006; Kopinke et al., 1996). Hilliou and co-workers could associate the lower T_g on non-purified samples to a plasticizing effect of the impurities, related to their influence on the ageing of the biopolymer. Additionally, in the referred work, the impurities were shown to

contribute to the thermal stabilisation, decreasing the melting temperature of the PHBV materials, as observed in the present work.

The crystallinity value also experienced a remarkable change in purified samples. The crystallinity value for the sample obtained with HAc and extracted with chloroform was 24%, but it increased to 50.1% when the chloroform-ethanol extraction method was used. This means that the crystallinity augmented in 108.8% when comparing the values for the HAc non-purified sample. A similar situation was observed when using HPr as substrate. The crystallinity was 10.5%when the biopolymer was extracted with chloroform, increasing to 18.4% when the chloroform-ethanol method was applied. This is a boost in crystallinity of 75.2% for the purified HPr sample regarding the value of the non-purified test. One hypothesis is that water was removed by the added ethanol. It has been reported that water can act as a plasticizer forming hydrogen bonds with the carbonyl groups (Sudesh et al., 2000) and, if water is removed, in situ crystallization can occur due to increased hydrogen bonding (Laycock et al., 2013) or even the rearranging of the biopolymer chains into lamellar crystals (Sudesh et al., 2000). Another hypothesis is that this change in the crystallisation values could be due to the presence of lipidic material that is not related with PHA when the extraction is done with chloroform (Ramsay et al., 1994), since some of these lipidic compounds could act as plasticisers.

The lipidic compounds present in the samples obtained using HAc and HPr as substrates were identified by GC-MS. The biopolymers analysed were extracted by means of the simple chloroform method and also the chloroform-ethanol. After this, they were methylated. The main difference was the presence of methylesters of hexa- and octa-decanoic acids in the sample extracted with chloroform. These compounds disappeared when the sample was extracted with the chloroform-ethanol method (Figure 7.2). These results confirm that the use of ethanol allows removal of lipid substances that were present in the unpurified biopolymer and thus changed the biopolymer properties.

The purification step is one of the factors that contribute to the high operation costs of the PHA production process. Results of this study show that properties of the biopolymer changed accordingly with the degree of purification undergone to the biopolymer. However, changes in properties do not preclude the application of the biopolymer. Indeed, the presence of some impurities may have a positive effect in some of the biopolymer properties. It might be possible to manipulate the

biopolymer properties not only by its monomeric composition but also by the presence of the identified impurities. On the other hand, the cost of the process will be potentially lower since a less purification degree will be required.

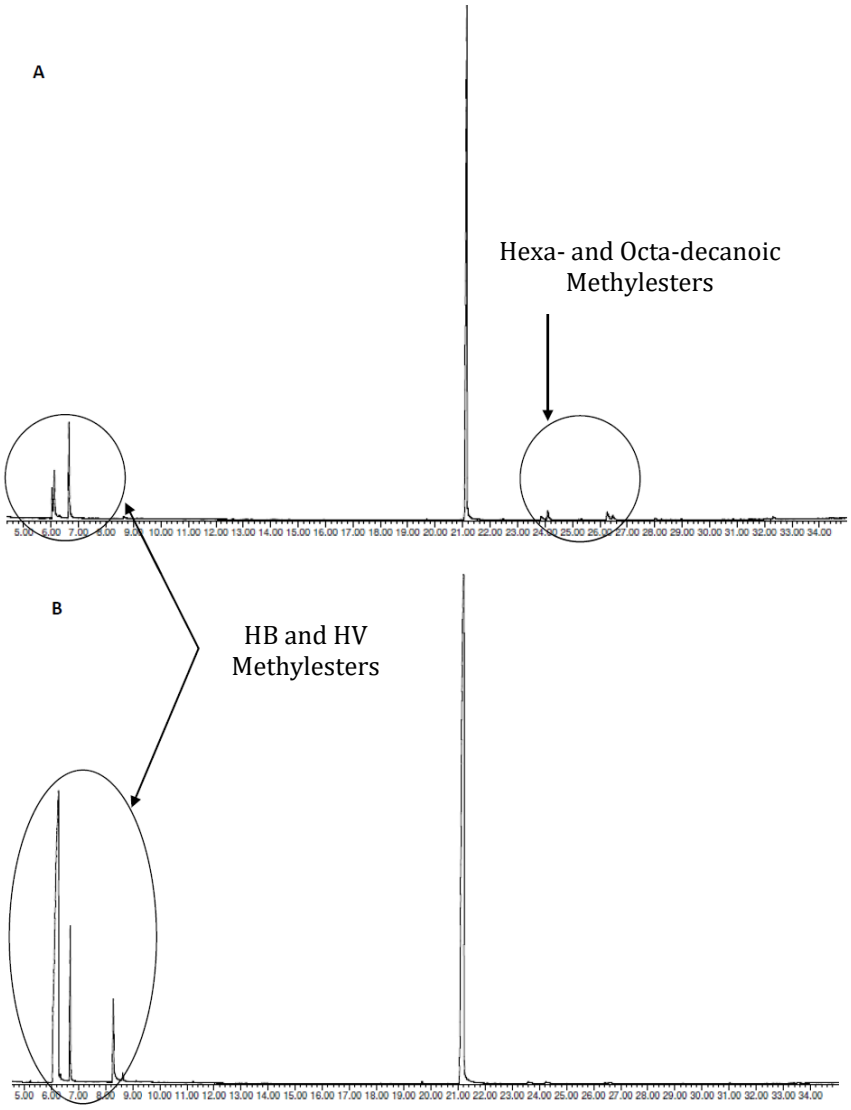


Figure 7.2. Results from the analysis by GC-MS of the sample using HAc as substrate. A. Sample extracted with chloroform. B. Sample extracted with chloroform-ethanol and concentrated.

7.5 CONCLUSIONS

This study showed that the physico-chemical properties of the biopolymer may be defined not only by the type of substrate, through the monomeric HB:HV distribution, but also the presence of salts in the medium or even the degree of purification imposed to the biopolymer.

- The type of substrate defined the HB:HV ratio, which was a good indicator of some of the properties when using the same MMC, as most of them are a function of the HB:HV ratio (i.e., T_g, T_m and M_w).
- The presence of NaCl in the reactor at a constant concentration of 21.6 g/L affected the HB:HV ratio and, consequently, affecting the thermal properties of the biopolymer as they depend on the %HB. Nevertheless, the M_w was affected in a higher extent as a result of the possible fact that the shorter chains were consumed while higher chains were produced, as a presumable consequence of the inhibitory effect of the NaCl.
- Less purification did not affect the M_w, while the crystallinity and T_g, T_m and T_d decreased maybe due to the presence of impurities which acted as plasticisers. Despite that the purity of the sample and the extraction method can significantly affect the properties of the biopolymer, combining a good extraction yield with a high purity may increase the extraction costs in a non-compensating way. The purification stage must be imposed mainly for applications that really need the absence of contaminating substances.

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Chapter 8

PHA PRODUCTION USING ORGANIC ACIDS OBTAINED FROM FISH-CANNING WASTEWATER

SUMMARY

The present chapter explores the ability of a mixed microbial culture (MMC) to produce polyhydroxyalkanoates (PHA) using an acidified fish-canning wastewater as nutrients and carbon source. The effect of the salinity (NaCl) on the PHA production process was explored.

The MMC was enriched in a sequencing batch reactor (SBR) fed in cycles with previously acidified wastewater diluted with tap water to have a composition of approximately 28.8:16.4:47.8:6.1 COD% of HAc:HPr:HBu:HVa, respectively; an ammonium content of 228.9 ± 47.7 mg NH_4^+-N/L; and a salinity of 3.19 ± 0.79 g NaCl/L. To know the maximum accumulation capacity of the MMC, two different types of accumulation assays were performed: (1) one experiment following the pulse-wise feeding method by adding pulses of substrate after its depletion and (2) another experiment applying a single feeding strategy at the beginning of the assay. The single pulse strategy showed a better performance since a productivity of 55.4 mg PHA/(L · h) was achieved in the batch accumulation assay while just 10.31 mg PHA/(L · h) were achieved when the pulse-wise feeding strategy was followed. In terms of kinetic and stoichiometric parameters, a solely pulse also showed better performance in comparison with the fed-batch accumulation experiments. Nevertheless, the high concentrations of NH_4^+ and NaCl during the accumulations negatively influenced the accumulation values. Biomass growth was relevant (0.366 and 0.266 Cmol X/Cmol VFA for the fed-batch and batch assays, respectively).

However, more research is needed regarding the excess of NH_4^+ and NaCl during the performance of the accumulation assays.

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8.1 INTRODUCTION

Fish-canning industries are essential for the local economy in Galicia (Spain), and they also present a significant role in the Spanish market. Just the Galician industries amount to 85% of the economic value of the whole national production of canned fish products and to 87% of the total volume (ANFACO-CECOPESCA, 2014). Even at European levels, the fish-canning companies and other marine industries are gaining importance in their manifest significance as key sectors for the economic growth of the European Union (European Commission-Executive Agency for Small and Medium-sized Enterprises, 2016). They make up the *Blue Economy* program, established by the European Commission. In fact, the *Blue Economy* represents about 5.4 million jobs although more growth is possible (European Commission-Maritime Affairs, 2016), as highlighted in the strategy proposed by the European Commission.

Beyond the employment generation and the economic growth related to fish-canning and marine industries, this sector produces great amounts of wastes that are not negligible and need further treatment. These effluents are mainly characterised by abundant flows with high organic loads in addition to high saline concentrations (Soto et al., 1990). The treatment of these saline wastewaters is usually accomplished by physico-chemical processes like coagulation, flocculation, filtration, etc. although the operational costs of this processes are elevated (Lefebvre and Moletta, 2006; Omil, 2003). In other cases, biological treatments are applied, which are chosen depending on the desired objective. In this case this saline effluents can be treated by aerobic or anaerobic biological systems (Feijoo et al., 1995; Gebauer, 2004; Hamoda and Al-Attar, 1995; Intrasungkha et al., 1999; Omil, 2003; Palmeiro-Sánchez et al., 2013; Uygur and Kargı, 2004).

Up to date, the applied treatments aimed at removing the pollutants from these kinds of effluents and preparing them to achieve appropriated quality for further discharge. However, recently these effluents are being considered as a source of organic matter to be used for the production of valuable compounds such as biopolymers. This new vision is in line with the concept of the *European Circular Economy* (European Commission-Environment, 2016). Instead of just accomplishing the treatment of these wastes, their valorisation can be a feasible option (Figure 8.1). For instance, agri-food waste streams are reported to be suitable substrates for PHA production (Nikodinovic-Runic et al., 2013). In this sense, fish-canning wastewaters seem to be also suitable for PHA production regarding their organic matter content,

although they have relevant NaCl concentrations (Soto et al., 1990) that can provoke negative effects.

Figure 8.1. Circular economy scheme: sustainability and valorisation of wastes for the obtainment of value-added products.

Several different residues have been already tested for PHA production in mixed microbial cultures: olive oil mill effluents (Dionisi et al., 2005), sugar cane molasses (Albuquerque et al., 2007; Albuquerque et al., 2010; Bengtsson et al., 2010; Duque et al., 2014), paper mill wastewater (Bengtsson et al., 2008; Jiang et al., 2012), waste activated sludge (Morgan-Sagastume et al., 2010; Morgan-Sagastume et al., 2015), tomato cannery (Liu et al., 2008), cheese whey (Duque et al., 2014), candy-bar industrial effluents (Tamis et al., 2014), etc. But unlike the fish-canning effluents, these effluents are characterised by the absence of significant amounts of proteins or sodium chloride which represent the main difficulties when treating this kind of wastes.

On the one hand, salinity is known to affect the PHA accumulation capacity (Palmeiro-Sánchez et al., 2016a) and the properties of the obtained biopolymer (Palmeiro-Sánchez et al., 2016b) in mixed microbial cultures. Although a positive

effect on the PHA storage has been observed by other authors using a pure culture of *Cupriavidus necator* (Passanha et al., 2014).

On the other hand, relevant concentrations of proteins lead to the presence of considerable amounts of ammonium in the medium of reaction. This essential nutrient, used by microorganisms to grow, is usually associated with lower accumulation percentages if compared with the accumulation under ammonium starvation conditions. Johnson et al. (2010) analysed the accumulation with no nitrogen source, under ammonium limitation conditions (C/N ratio of 40 Cmol/Nmol) and ammonium excess conditions (C/N ratio of 8 Cmol/Nmol). These authors found that PHA content was significantly low at 8 Cmol/Nmol and they also realised that the biopolymer production rates slowed down when higher ammonium amounts were available (Johnson et al., 2010). Furthermore, Moralejo et al. (2013) performed two experiments at different C/N ratios of 76 and 770 Cmol/Nmol. These authors found that the maximum accumulation did not differ in both experiments since the ammonium was not consumed in the first hours of the storage assays because storage was promoted over growth (Moralejo-Gárate et al., 2013). Johnson et al. (2010) and Moralejo et al. (2013) observed opposite behaviours when dealing with an excess of ammonium in the accumulation assays, which indicates that further research is needed.

As aforementioned, effluents from the tuna-canning industry are good candidates to act as organic carbon source to produce (PHA) due to their composition and to the relevance of this sector in Galicia. Its significance is undeniable since 47 out of 67 fish-canning companies are dedicated to the processing of tuna products, which represents the 70% of the Spanish tuna-canned products (ANFACO-CECOPESCA, 2014). The challenge of using this substrate for PHA production relies on the fact that it contains potential inhibitors, which can be detrimental for the process.

8.2 OBJECTIVES

The principal aim of the present study is to investigate the feasibility of PHA production using an acidified fish-canning wastewater, containing relevant concentrations of volatile fatty acids, ammonium and sodium chloride. To the knowledge of the authors, no previous research has been done regarding PHA

production using high saline and high NH_4^+ content. So, the specific objectives of this study are:

- To study the performance of the enrichment of a PHA-accumulating mixed microbial culture (MMC) using as organic carbon source the effluent from the acidification reactor operated in Chapter 4 treating the effluent from tuna cookers. The influence of the high concentrations of NaCl and NH_4^+ over the biomass selection process will be also evaluated.
- To evaluate the PHA-accumulation capacity of the MMC by the application of two feeding strategies: pulse feeding or a single initial feeding addition.

The results obtained of the biopolymer accumulation were evaluated in terms of the calculated kinetic and stoichiometric parameters.

8.3 MATERIALS AND METHODS

8.3.1 Experimental set-up

A system of three stages (Figure 8.2) was designed to produce biopolymers using acidified fish-canning wastewater as feedstock.

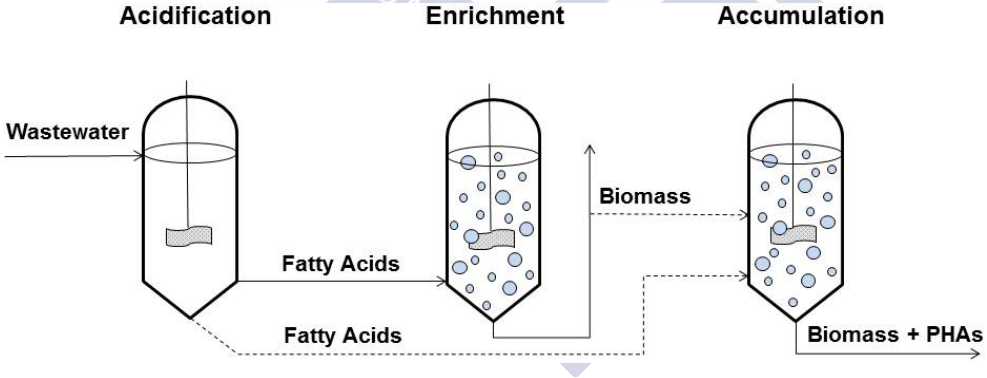

Figure 8.2. Layout of the three stages for PHA production.

The first reactor was used for the acidification of a fish-canning effluent (Acidification Reactor) with the aim of producing volatile fatty acids. These organic acids were used as a substrate in the following two stages. The second reactor was used for the selection of a microbial mixed culture (Enrichment reactor) which would be able to store a high amount of PHA inside the cells. The third reactor was used for the maximization of biopolymer content inside the cells (Accumulation Reactor) using the same acidified effluent.

8.3.1.1 Acidification Reactor

The acidification reactor (RWC) was a semi-continuous reactor (Álamo, Spain) fed with tuna cookers effluent and described in section 4.3.1.2 of Chapter 4 "Production of Organic Acids from Fish-canning Wastewaters".

8.3.1.2 Enrichment Reactor

A sequencing batch reactor (SBR) (Álamo, Spain) of 1.8 L of useful volume (Figure 8.3, left) was used for the selection of a MMC able to store PHA.

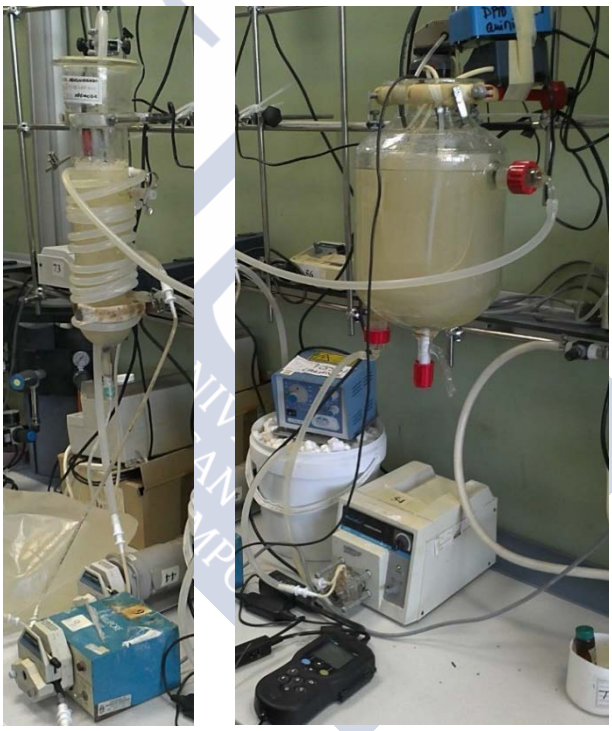

Figure 8.3. Enrichment (left) and accumulation (right) reactors.

The pH value was measured on-line and registered at the end of the famine phase with a membrane electrode (Crison, Germany). Air was supplied to the reactor through an air diffusor so as to maintain the dissolved oxygen (DO) concentration above approximately 1 mg O_2/L. The DO concentration was measured and acquired with an oxygen meter provided with a membrane sensor (Hach-Lange, USA). The

reactor was jacketed, so the temperature was maintained at 30 °C with a thermostatic water bath (Techne Inc., USA).

8.3.1.3 Accumulation Reactor

The accumulation reactor (Álamo, Spain) is a batch or fed-batch reactor of variable volume (Figure 8.3, right). It was used to maximize the PHA in the cells.

The pH value was not controlled although it was measured and registered with a membrane electrode (Crison, Germany) throughout the accumulation experiment. The temperature was controlled at 30 °C by using a thermostatic bath (Techne Inc., USA). The DO concentration was measured and acquired during the entire accumulation assay by using an oxygen meter provided with a membrane sensor (Hach-Lange, USA). It was operated under non-sterile conditions.

8.3.2 Operational Conditions

8.3.2.1 Acidification Reactor

Detailed information about the operation of the acidification reactor treating the effluents from a tuna cooker is provided in Chapter 4, where the results of its performance are included. A brief view summary of the composition of the acidified effluent is supplied in Figure 8.4.

8.3.2.2 Enrichment Reactor

The enrichment SBR was inoculated with biomass collected from another enrichment reactor treating synthetic wastewater, as described in Chapter 5, and fed with the diluted effluent of the acidification reactor.

The SBR was operated for 288 days at an organic loading rate of 2.73 ± 0.32 g COD/(L · d) with the effluent of the acidification reactor (Figure 8.4). The SBR was operated cycles of 12-hours at a hydraulic and solids retention time (HRT and SRT) of 24 hours. The SBR operates under aerobic conditions throughout the whole operational cycle, which was distributed in four phases: (1) the feeding of the acidified fish-canning effluent supplied with allylthiourea (ATU), at a concentration of 2 mL ATU/L, to avoid nitrification activity (15 min); (2) the reaction phase when the aerobic dynamic feeding (ADF) is applied and the feast/famine regime take place (675 min); (3) the withdrawal of half of the useful volume of the reactor (0.9 L) as

effluent (15 min); and, finally, (4) an idle phase (15 min). The SBR was operated under non-sterile conditions and without pH control. The performance of the enrichment reactor was monitored twice a week. Liquid and solid samples were taken at the end of the cycle to monitor the pH, VSS, TSS, and ammonium concentrations at the end of the famine phase, which corresponds to the beginning of the next cycle.

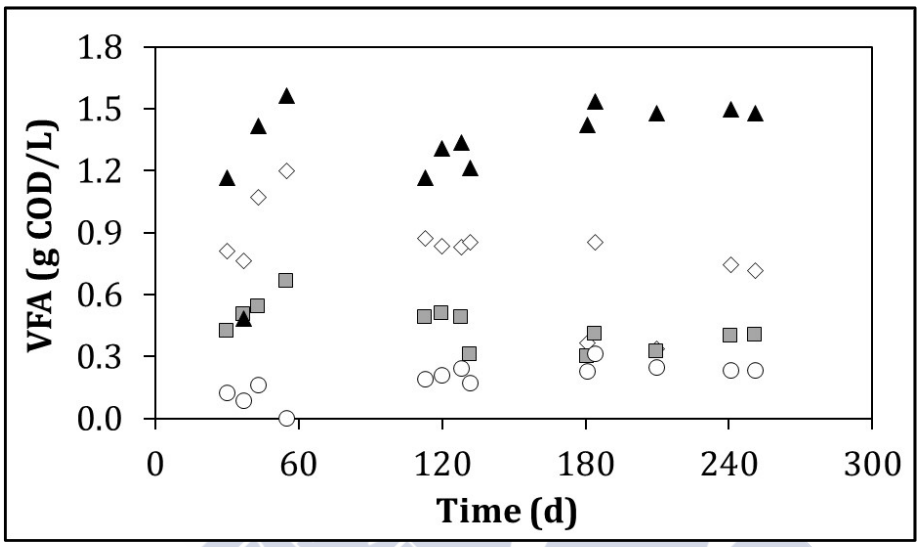

Figure 8.4. Concentrations (g COD/L) of the different VFAs in the feeding of the enrichment reactor: Acetic (▲); Propionic (□); Butyric (◇); and Valeric (○) acids.

8.3.2.3 Accumulation Reactor

The accumulation reactor was inoculated with biomass withdrawn from the enrichment reactor at the end of the SBR cycle. The accumulation experiments were performed using as a substrate the effluent of the acidification reactor. These assays are characterised by the presence of high concentrations of ammonium and sodium chloride. Due to the fact of the presence of a nutrients source, a competition between growth and accumulation for the use of the carbon source is expected.

Two different types of accumulation assays were performed: (1) one operated in fed-batch mode and (2) another operated in batch mode.

1. Fed-batch assay
   Substrate was added in pulses. Exhaustion of the VFAs present in the feeding was noticed by the increase of the DO concentration, which was monitored and measured on-line. Each time DO concentration rose up, a new pulse of feeding was added. The volume of each pulse was of 40 mL with a concentration of 26.17 g COD_{VFA}/L containing 28.8:14.4:48.8:8.0 of HAc:HPr:HBu:HVa in COD%. To calculate the carbon balances, these values were transformed into Cmmol units, which corresponded to 28.3 Cmmol VFAs per pulse of 40 mL.
2. Batch assay
   One single volume of 520 mL of substrate was added. The concentration was of 17.7 g COD_{VFA}/L with a composition of 17.1:9.7:64.2:9.0 of HAc:HPr:HBu:HVa in COD%. The carbon balances were calculating by converting these values into Cmmol units, which meant a solely pulse of approximately 250 Cmmol VFA.

Data collected from all these accumulation experiments was used to calculate the corresponding stoichiometric and kinetic parameters of the maximum accumulation performance of the enriched MMC.

8.3.3 Analytical Methods

The concentrations of Total and Soluble Chemical Oxygen Demand (COD_T and COD_S, respectively) were determined following the method 5220C of the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005) modified for samples with high concentration of salinity (Soto et al., 1989). The concentrations of VFAs were measured following a the method described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005) by gas chromatography (GC) (Hewlett Packard 5890A, USA). The concentrations of total ammonium (NH_4^+) were determined spectrophotometrically (Bower and Holm-Hansen, 1980) and those from other ions, like sodium (Na^+), potassium (K^+) and sulphate (SO_4^{2-}), by ionic chromatography (IC). The pH was measured with an electrode (Crison Instruments, USA) and the temperature with a digital thermometer. The DO concentration was measured with an oxygen pocket meter (Hach-Lange, USA).

The concentrations of Total (TSS) and Volatile Suspended Solids (VSS) were determined according to the methods described in the *Standard Methods for the Examination of Water and Wastewater* (APHA-AWWA-WPCF, 2005). PHAs inside the cells were analysed by a chromatographic method (Smolders et al., 1994).

Operational cycles of the enrichment reactors were characterised by monitoring the values of different parameters throughout single operational cycles. Liquid and biomass samples were taken at various times to monitor the progression of pH, temperature, DO, VFAs, VSS, TSS, ammonium and PHA concentration profiles. Similar procedure was followed in the case of the PHA accumulation assays. Data collected from the enrichment cycles and accumulation experiments were used to calculate the stoichiometric and kinetic parameters corresponding to the accumulation performance of the microbial mixed culture.

All these methods are described in detail in Chapter 2 "Materials and Methods".

8.3.4 Calculations

Calculations of the kinetic and stoichiometric parameters corresponding to PHA production and carbon balances are performed with the experimental results obtained from the monitoring of enrichment cycles and accumulation experiments. The amount of PHA, active biomass, CO_2, rates and also the yields for each produced/consumed compound were determined in each enrichment and/or accumulation batch assay. For these calculations, the units are expressed in carbon moles. All these calculations are described in detail in Chapter 2 "Materials and Methods".

8.4 RESULTS AND DISCUSSION

8.4.1 Enrichment of a MMC with acidified fish-canning wastewater

The enrichment reactor was monitored so as to observe the evolution of the performance of the selected microbial community. Enrichment cycles and accumulation assays were monitored to provide information about the effects caused by the presence of sodium chloride and the ammonium excess contained in the acidified wastewater.

The average TSS and VSS concentrations inside the reactor were 1.14 ± 0.28 g TSS/L and 1.03 ± 0.25 g VSS/L, respectively. The 90.5 ± 5.2 % of the TSS were due to

the VSS. The average values of other measured parameters were 281.1 ± 57.1 mg N-NH_4^+ - N/L, 3.19 ± 0.79 g NaCl/L at the end of the famine phase.

Several enrichment cycles were monitored, which provided data similar to that shown in Figure 8.5, considered as a cycle representative of the reactor performance.

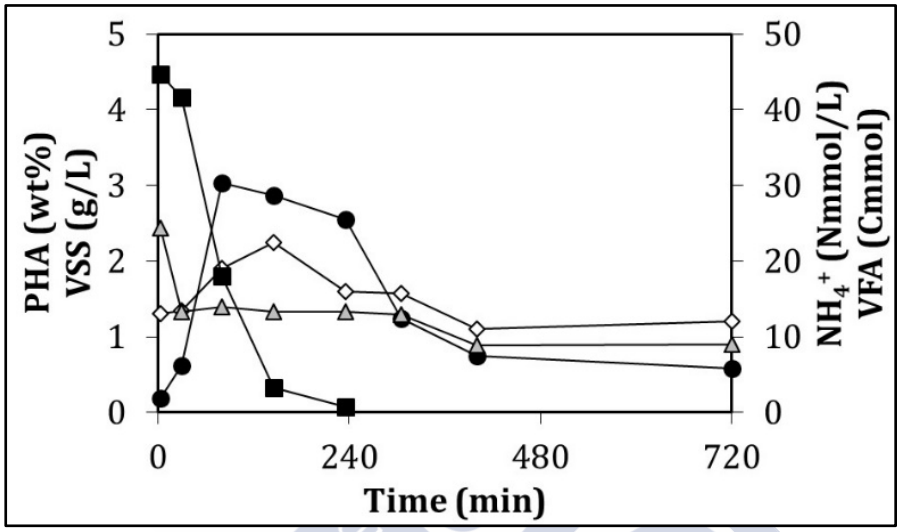

Figure 8.5. Evolution of the measured compounds during a representative enrichment cycle performed on the day 261 of operation. Amount of consumed VFAs (Cmmol) (■); NH_4^+ - N concentration (Nmmol/L) (△); VSS concentration (g/L) (◇); and percentage of acummulated PHA (wt%) (●).

Estimated yields for biomass and CO_2 were of 0.351 Cmol X/Cmol VFA and 0.366 Cmol CO_2/Cmol VFA, respectively. These high yields demonstrate that biomass growth was the most relevant process in the system as the yields for PHA production were of 0.131 Cmol HB/Cmol VFA and 0.006 Cmol HV/Cmol VFA for each one of the monomers. The estimated error of the balances was of 14.7%.

The specific substrate consumption rate was of 0.183 Cmol VFA/(Cmol X · h) and the specific biopolymer production rate was of 0.025 Cmol PHA/(Cmol X · h). The difference between both rates corresponds to the growth favoured over accumulation during the enrichment cycle. The specific growth rate was of 0.064 Cmol/(Cmol X · h) and the specific CO_2 production rate was of 0.067 Cmol CO_2/(Cmol X · h). Nevertheless, Duque et al. (2014) obtained better results when using

fermented molasses and fermented cheese whey as feedstocks, with values of 0.38 Cmol VFA/(Cmol X · h) and 0.25 Cmol PHA/(Cmol X · h) for substrate consumption and PHA production specific rates, respectively, for fermented molasses and values of 0.34 Cmol VFA/(Cmol X · h) and 0.27 Cmol PHA/(Cmol X · h) for substrate consumption and PHA production specific rates, respectively, for fermented cheese whey. Duque et al. (2014) explained their better results in base to the application of 8 cycles per SRT since the selective pressure is increased when lower number of cycles per SRT are done (Jiang et al., 2011). Nonetheless, Jiang et al. (2011) used a SRT equal to the HRT of 1 day, the same used in the present work, while Duque et al. (2014) applied a HRT of 1 day and a SRT of 4 days. In this way, it seems that the HRT also have an influence in the selection of the MMC since in the present study 2 cycles were done per SRT and the MMC enrichment values were low in comparison with the ones obtained by Duque et al. (2014).

8.4.2 Fed-batch accumulation with acidified fish-canning wastewater

Experiments to evaluate the PHA accumulation capacity of the biomass were performed by applying the substrate in pulses throughout the assay (fed-batch) or as one single pulse at the beginning of the experiment (batch). These feeding strategies were evaluated with a substrate rich in VFAs which have been recognized as inhibitory for the accumulation, but maintained at neutral pH. Furthermore, the impact of the presence of NaCl in the media was studied, as its concentration was constant in the batch assay but it continuously increased in the fed-batch one.

Since, in the fed-batch assay, the substrate was fed in pulse-wise feeding, the exhaustion of the substrate was noticed by the increase of the DO concentration, which was monitored and measured on-line (Figure 8.6). In this fed-batch experiment, each pulse corresponded to a volume of feeding of 40 mL with a concentration of 26.17 g COD_{VFA}/L with a VFA composition of 28.8:14.4:48.8:8.0 of HAc:HPr:HBu:HVa, in COD%. This means that in each 1.05 g COD_{VFA} added per pulse were present 0.302 g COD due to HAc; 0.151 g COD due to HPr; 0.512 g COD due to HBu; and 0.084 g COD due to HVa. Twenty pulses were added to reach a final net amount of VFA added of 568.9 Cmmol VFA after the 27 h of the accumulation assay.

After monitoring the concentrations of the parameters measured throughout the fed-batch assay (Figure 8.7) the VSS and TSS concentration increased from 0.56 to 2.5 g VSS/L and from 0.73 to 3.3 g TSS/L, respectively. The growth of active

biomass (X) was relevant as nitrogen was added with each pulse with a specific production rate of 0.07 Cmol X/(Cmol X · h). The concentration of NH_4^+ in the assay increased from 0.51 to 45.1 mM. The maximum obtained biopolymer content was about 8.4 wt% of PHA after 27 h of reaction. In productivity terms, this means a value of 10.3 mg PHA/(L · h) (Table 8.1).

Due to the observed growth values and the low values of accumulation percentages, it can be assumed that the competition between biomass growth and PHA accumulation occurred. Typically, accumulation assays are performed without nitrogen addition to avoid biomass growth which hinders the PHA accumulation. As an example Johnson et al. (2010) observed that PHA accumulated was as low as 8 Cmol/Nmol in the presence of ammonium. In the present study, the VSS augmented from 0.56 to 2.50 g VSS/L (Figure 8.7) during the accumulation experiment. This difference between final and initial VSS concentration is attributed to PHA accumulation and biomass growth. However, in the present case, the accumulation percentage increased only from 4.54 to 8.35 wt% of PHA due to the biomass growth. Moreover, kinetic and stoichiometric parameters support this statement since PHA specific production rates (0.005 Cmol HB/(Cmol X · h) and 0.001 Cmol HV/(Cmol X · h)) are very low in comparison with growth rates (0.068 Cmol X/(Cmol X · h)). Accumulation yields were much higher for growth (0.366 Cmol X/Cmol VFA) than for the PHA accumulation (0.029 Cmol HB/Cmol VFA and 0.005 Cmol HV/Cmol VFA).

Not only the presence of ammonia but also the presence of sodium chloride is known to affect the microbial accumulating response of the system. Values of IC_{50} of approximately 5 g NaCl/L were reported for PHA accumulation in similar enriched MMCs (Palmeiro-Sánchez et al., 2016a). In the present case, the salt concentration ranged from 2.2 to 8.3 g NaCl/L, indicating that a possible inhibition could take place influencing the PHA storage.

The PHA accumulation rate was only 0.006 Cmol PHA/(Cmol X h) (Table 8.2). It can also be observed that HB accumulation was promoted over HV accumulation. This behaviour might be explained by the fact that propionate has higher energy potential production in comparison with acetate when cell maintenance comes along during inhibiting processes, as it was observed in previous studies (Palmeiro-Sánchez et al., 2016a). This statement is based on the assumption that butyrate and valerate are not expected to contribute decisively to biomass maintenance (Pardelha et al., 2014).

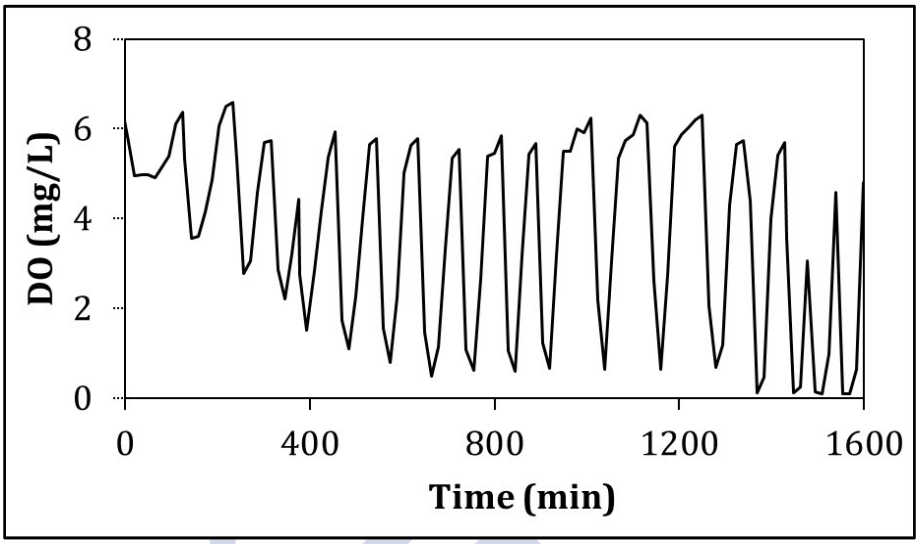

Figure 8.6. Profiles of dissolved oxygen concentrations throughout a fed-batch accumulation assay performed with biomass collected from the enrichment reactor on day 211.

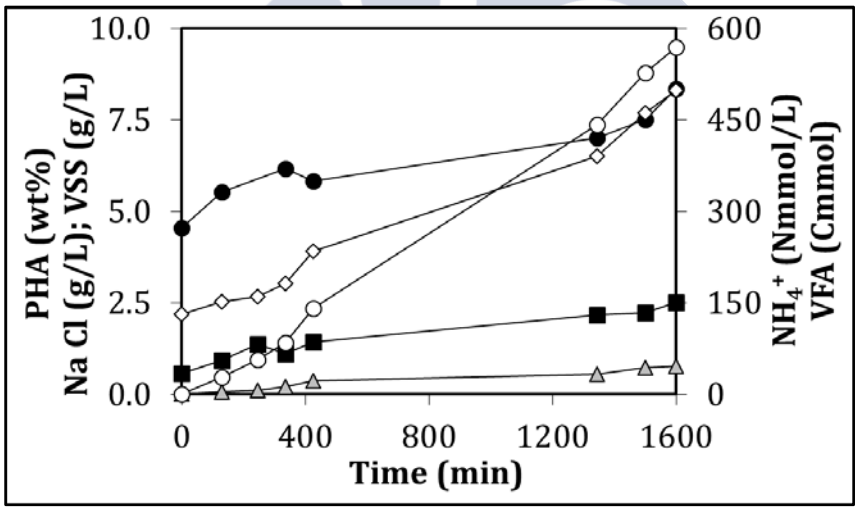

Figure 8.7. Evolution of the concentrations of the measured parameters in the fed-batch accumulation assay (day 211). Cumulative amount of consumed VFAs (Cmmol) (○); NH_4^+-N concentration (Nmmol/L) (△); VSS concentration (g/L) (■); NaCl concentration (g/L) (◇) and percentage of the stored PHA (wt%) (●).

8.4.3 Batch accumulation with acidified fish-canning wastewater

The substrate was fed by adding just one single pulse of substrate with a concentration of 17.7 g COD/L with a VFA composition of 17.1:9.7:64.2:9.0 of HAc:HPr:HBu:HVa, in COD%, which was completely consumed after 6 hours. The evolution of the DO concentration was monitored and measured on-line (Figure 8.8).

The batch assay lasted 24 hours although the maximum value of 5.7 wt% PHA was achieved after 2.7 hours of the experiment. At this moment, 122 Cmmol VFA had been consumed (Figure 8.9). After this point, the PHA inside the cells started to diminish (Figure 8.9) even though half of the added VFAs were still present in the media amounting to approximately 125 Cmmol VFA.

Table 8.2 gives information about the values of several of these parameters. The VSS and TSS concentration increased from 0.87 to 1.19 g VSS/L and 1.71 to 2.59 g TSS/L, respectively. It is important to remark that the growth of active biomass (X) was relevant again at a specific rate of 0.121 Cmol X/(Cmol X · h). The concentration of ammonium in the assay decreased from 75 to 56 Nmmol/L in the accumulation period and it also varied from 75 to 10 Nmmol/L during the whole cycle.

In productivity terms, the accumulation value of 5.7 wt% is related to a value of 55.4 mg PHA/($L_{reactor}$ · h). The composition of the obtained biopolymer was of 15.9 g/g in terms of HB:HV ratio (Table 8.1), which was very similar to the value at the beginning of the assay (HB:HV of 15.3 g/g). In this case, both biopolymers were produced in a similar extent. This fact could be due to the constant concentration of 6.81 g NaCl/L throughout the cycle as the microorganisms were affected more or less in the same grade. After the point of maximum accumulation both monomers were consumed for maintenance. After 6 hours from the beginning of experiment, the HB:HV ratio was approximately 26 g/g, while after 24 hours this value increased tremendously as HV was fully depleted (Table 8.1).

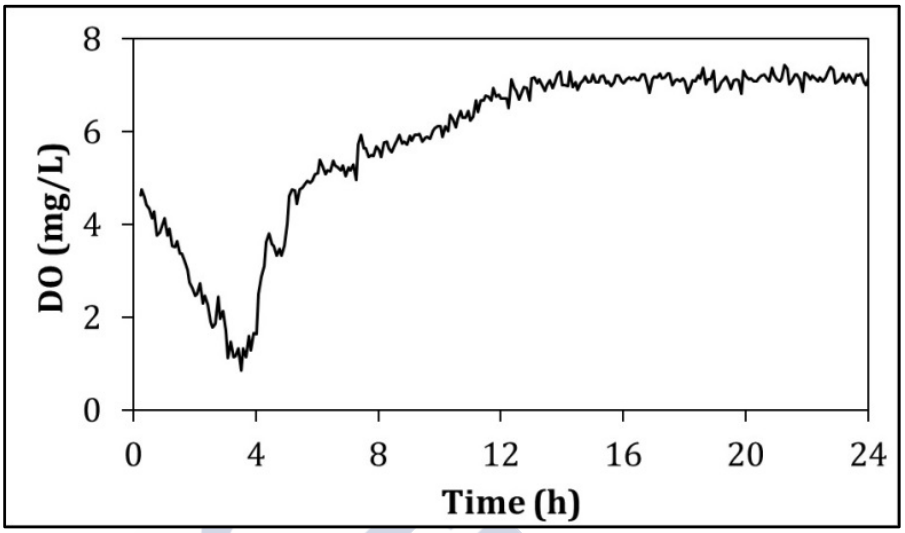

Figure 8.8. Profiles of dissolved oxygen concentrations throughout a batch accumulation assay performed with biomass collected from the enrichment reactor on day 260.

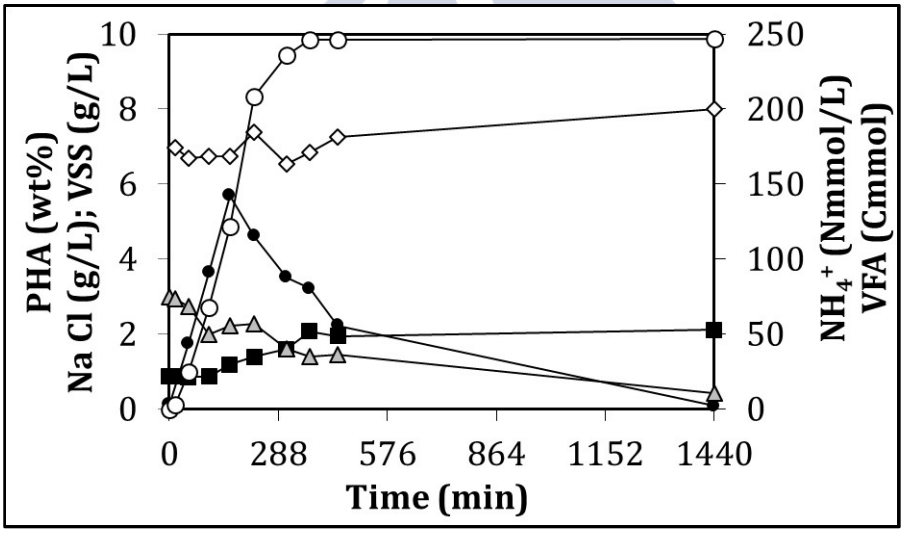

Figure 8.9. Evolution of the concentrations of the measured parameters in the batch accumulation assay performed with biomass collected from the enrichment reactor on day 260 of operation. Consumed VFAs (Cmmol) (○);NH_4^+-N concentration (Nmmol/L) (△); VSS concentration (g/L) (■); NaCl concentration (g/L) (◇) and percentage of the stored PHA (wt%) (●).

Table 8.1. Summary of the percentage of accumulation, concentrations, and PHA productivity of the accumulation assays.

Experiments	HB wt%	HV wt%	PHA wt%	HB:HV (g/g)	Productivity (mg PHA/L h)	TSS (g/L)	VSS (g/L)
Fed-batch (0 h)	2.21	2.33	4.54	0.95	-	0.72	0.56
Fed-batch (27 h)	7.14	1.21	8.35	5.91	10.31	3.29	2.50
Batch (0 h)	0.12	0.01	0.13	15.3	-	1.71	0.87
Batch (2.7 h)	5.36	0.34	5.70	15.9	55.4	2.59	1.19
Batch (24 h)	0.09	0.00	0.09	-	-	3.66	2.12

Table 8.2. Summary of the main stoichiometric and kinetic parameters obtained on the accumulation assays.

Experiments	$-q_{VFA}$ Cmol/ $Cmol_X$ h	q_{HB} Cmol/ $Cmol_X$ h	q_{HV} Cmol/ $Cmol_X$ h	q_X Cmol/ $Cmol_X$ h	q_{CO2} Cmol/ $Cmol_X$ h	Y_{HB} Cmol/ $Cmol_{VFA}$	Y_{HV} Cmol/ $Cmol_{VFA}$	Y_X Cmol/ $Cmol_{VFA}$	Y_{CO2} Cmol/ $Cmol_{VFA}$
Fed-batch	0.187	0.005	0.001	0.068	0.064	0.029	0.005	0.366	0.345
Batch	0.536	0.067	0.004	0.121	0.372	0.125	0.008	0.226	0.694

All values correspond to the point of maximum accumulation measured in the experiment.

8.4.4 Influence of the feeding strategy on the PHA-accumulation

When VFAs are used as substrate is common to perform accumulation experiment fed in pulses to avoid the possible substrate inhibition by substrate (Serafim et al., 2004). In these cases, the pulse concentration is fixed in conservative values (about 20 Cmmol/L). However, no information regarding the effects of the high VFAs concentrations is available, and in special in the case or effluents containing also ammonia and NaCl.

In the present study, specific features associated to the use of fish-canning wastewater must be considered. When pulse wise feeding (pulses of 40 mL) is applied a progressive increase of the concentrations of NaCl and NH_4^+ simultaneously occur. However, when the feeding was supplied in a one single pulse, the ammonium concentration progressively decreased due to biomass growth and the NaCl concentration remained constant throughout the experiment.

In general terms, it can be said that the addition of the whole substrate at the beginning of the accumulation experiment (batch assay) provided better results than its addition in pulses (fed-batch assay) regarding the productivity but also the yield values. The most significant and relevant data is the PHA productivity, which is related to the economic feasibility of the process. The productivity was of 10.31 mg PHA/(L h) for the fed-batch assay while it was 55.4 mg PHA/(L h) for the batch assay (Table 8.1), which means that it was more than 5 times higher. Duque et al. (2014) obtained productivities between 150 – 560 mg PHA/(L · h). These values are much higher than those obtained in the present study. This could be due to the inhibitory effect of the NaCl and the presence of large ammonium concentrations, which enhances the biomass growth. Having a look at Table 8.3, the obtained accumulation rates (0.006 Cmol PHA/(Cmol X · h) and 0.071 Cmol PHA/(Cmol X · h)) are quite low in comparison with other authors, that obtained values in the range of 0.15 – 0.47 Cmol PHA/(Cmol X · h) for real fermented wastewaters like cheese whey or sugar cane molasses, where no inhibitors or excess of nutrients were present (Albuquerque et al., 2011; Duque et al., 2014). With respect to the salt an IC_{50} of approximately 5 g NaCl/L for PHA accumulation has been reported (Palmeiro-Sánchez et al., 2016a) and the concentration in the present study was at maximum 8 g NaCl/L in both accumulation experiments, which supports the idea of having NaCl inhibition in both accumulations.

The yield of the process was also improved when operating in one single pulse mode. The batch experiment beat the fed-batch one as the yields were significantly higher for both monomers, with values of 0.029 Cmol HB/Cmol VFA and 0.005 Cmol HV/Cmol VFA for the fed-batch assays and 0.125 Cmol HB/Cmol VFA and 0.008 Cmol HV/Cmol VFA. Nonetheless, the most relevant change was observed for the HB yield while the HV yield remained nearly constant. This can be attributed to the presence of NaCl, since it presence promotes the HB accumulation over the HV accumulation since propionate leads to higher energy release (Palmeiro-Sánchez et al., 2016a). It is usual to find low yield values when fed-batch experiments are performed without

nutrient limitation. Values of 0.08 Cmol HB/Cmol VFA (Bengtsson et al., 2008) were obtained under nutrient excess using fermented papermill wastewaters as substrate.

The exhaustion rate of substrate was 0.187 Cmol VFA/(Cmol X h) for the fed-batch while this value was 0.536 Cmol VFA/(Cmol X h) for the batch assay. Values of 0.191 Cmol VFA/(Cmol X · h) and 0.18 Cmol VFA/(Cmol X · h) were respectively obtained for the consumption of fermented paper-mill wastewaters (Bengtsson et al., 2008) and fermented molasses (Duque et al., 2014). These results were obtained for fed-batch accumulation experiments with fermented substrates, which were similar to the values obtained for the fed-batch experiment in the present study. Nevertheless, the specific substrate consumption rates for synthetic mixtures of VFAs under nutrient excess are higher when compared to those from fermented substrates. Values in the range of 0.4 – 1.4 Cmol VFA/(Cmol X · h) have been reported (Beccari et al., 1998; Dionisi et al., 2004). This can be attributed to the better performance of the enrichment with synthetic substrates due to the control of the feeding composition and also the absence of inhibitory compounds in the medium.

As indicated previously, the operation of the accumulation assays was marked for the excess of NH_4^+ due to the use of fermented fish-canning wastewaters. Bengtsson et al. (2008) obtained better results for the nutrients limitation case, since 42.7 wt% of PHA was achieved under nutrient starvation conditions while 31.9 wt% of PHA were obtained under nutrient excess (Bengtsson et al., 2008). Contradictory results were obtained by other authors: Johnson at al. (2010) observed that higher C/N ratios led to better accumulation performance while Moralejo et al. (2013) stated that the C/N ratio did not influenced the maximum biopolymer accumulation once an appropriated enrichment is produced under nitrogen limiting conditions. The first authors used acetate as feeding supplied in pulses while Moralejo et al. (2013) used glycerol as substrate fed in a one single pulse. None of the authors considered the influence of the feeding regime in their experiments. However, looking at the accumulation and growth yields obtained in the present study, it can be observed that the feeding strategy influences the accumulation performance. Growth is promoted in the fed-batch experiment since a higher yield value (0.366 Cmol X/Cmol VFA) and lower accumulation value (0.034 Cmol PHA/Cmol VFA) are obtained in comparison with the batch experiment (0.226 Cmol X/Cmol VFA and 0.133 Cmol PHA/Cmol VFA). This means that less substrate was directed to growth when a solely pulse with a high VFA concentration is added to the reactor and, subsequently, less competition between biomass growth and biopolymer

accumulation would be expected. In any case, in order to avoid troubling competition between growth and accumulation, more experiments based in one single VFA pulse should be performed so as to establish the optimal accumulating conditions for the obtainment of biopolymers under excess of nutrients.

Summing up, it is better to feed in one single pulse than in pulse wise feeding since higher productivities and higher yields are achieved, as it has been previously discussed in this Section. In any case, further research is needed at this point to improve the results. For example, an enrichment operating under saline conditions is expected to provide better productivity values and to influence the composition of the obtained biopolymers. In general, it can be said that the enriched MMC is expected to be the key factor so as to improve the kinetic and stoichiometric parameters in the subsequent accumulation stage. Then, another key point is to study the influence of the cycle length in relationship with the C/N ratio. This is expected to improve the MMC enrichment, which further will also improve the accumulation of biopolymer and productivity of the process.

8.5 CONCLUSIONS

A MMC was enriched using a mixture of VFAs obtained from the acidification of fish-canning wastewaters at 3.19 ± 0.79 g NaCl/L.. Using this biomass, two different accumulations were performed: fed-batch and batch assays. Results from the accumulation experiments indicated that higher percentages were obtained in the fed-batch (8.35 wt%) in comparison with the batch (5.70 wt%). Nevertheless, the addition of VFAs in a single pulse provides higher productivities, with values of 55.4 mg PHA/(L · h) in the batch assay in comparison with 10.31 mg PHA/(L · h) in the fed-batch assay. It is also important to remark that biomass growth was more favoured when the pulse-wise feeding strategy was applied, with a biomass yield of 0.366 Cmol X/Cmol VFA in comparison with the value of 0.226 Cmol X/Cmol VFA obtained when one single pulse was applied.

Low kinetic and stoichiometric values were obtained for the accumulation experiments when comparing with other authors due to the fact that they were performed with salt concentrations of 8 g NaCl/L. One indicator of this inhibition is the low HV production (Table 8.1) since propionic and valeric acids were present in the feeding (Figure 8.4) and the HB content increased while the HV amount seemed to decrease with time.

Another reason for the low kinetic and stoichiometric values for PHA production can be attributed to the presence of high concentrations of NH_4^+, with yields for biomass growth of 0.366 and 0.266 Cmol X/Cmol VFA for the fed-batch and batch assays, respectively. The suspended solids concentration also corroborates this, with increasing values from the beginning of the experiment to the moment when the maximum concentration of PHA was achieved. Values from 0.56 to 2.50 g VSS/L and 0.87 to 1.19 g VSS/L were achieved for the fed-batch and batch assays, respectively. Regarding the competition between growth and accumulation, the feeding regime also affected the amount of PHA stored and the HB:HV ratio, which in a further extent also affects the properties of the biopolymers.

Nevertheless, in global terms, results must be optimised to obtain a better enrichment which will subsequently improve the accumulation.

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General Conclusions

The main conclusions of this work, focused on the obtainment of value-added products (biogas, volatile fatty acids and polyhydroxyalkanotes) from saline wastewaters, are described below:

Methane production under saline conditions

Aerobic granular sludge (AGS) and flocculent sludge (FLAS) were successfully treated by an anaerobic reactor under saline conditions (2.1-5.2 g Na^+/L).

Biodegradability (BD) results were similar for both AGS and FLAS, with average values of 32% and 27%, respectively, indicating that the aggregation state of the biomass did not influence the performance of the process. In addition, the BD value for AGS under brackish conditions (32%) was very close to the BD value for AGS without the presence of NaCl in batch assays (33%).

The specific methanogenic activity (SMA) of the biomass was determined under non-brackish conditions with a value of 0.016 L CH_4/(g VS · d). Afterwards, the SMA was determined for the biomass treating the AGS under saline conditions and the result was 0.015 L CH_4/(g VS · d). It seems that the anaerobic biomass was not influenced by operating under these saline concentrations (2.1-5.2 g Na^+/L).

No inhibition due to ammonia was observed since free ammonia (FA) concentration was between 8.1 and 9.9 mg NH_3-N/L, which is lower than the inhibitory value of 20 mg NH_3-N/L.

Sulphide concentrations in the reactor were of 38 - 93 mg/L and, in the biogas, were of 1.5 – 3.8%. No inhibition was observed in the liquid phase although the presence of sulphide in the biogas limits its further applications.

Methane content was between 56 – 62 % in the biogas, which are in the range of values for sewage anaerobic digestion (55 - 65%).

Production of organic acids from fish-canning wastewaters

Volatile fatty acids (VFAs) were produced using two different fish-canning wastewaters: one from the washing of the tuna boilers (WW) and other directly from the tuna boilers (WC). These wastewaters are characterised by the high content in proteins and NaCl. The reactor treating WW was an SBR with a hydraulic retention time HRT of 1 day, and the reactor treating WC was a semi-continuous reactor with a HRT of 2 days.

In terms of COD, conversions of 63 % for WW and nearly 87 % for WC were achieved. The VFA composition obtained when acidifying WW was 45.5:27.7:9.1:17.7 COD% of HAc:HPr:HBu:HVa while these values were of 43.1:12.6:38.4:5.9 COD% of HAc:HPr:HBu:HVa when treating WC. Acetic was the most produced acid in both cases. Nevertheless, when treating WW propionic was the second VFA mainly produced while butyric was the second one most produced when treating WC. This could be due to the presence of higher concentrations of NaCl, since it promotes the production of butyric acid. It can also be explained by the higher HRT since it also promotes the production of longer VFAs.

The presence of NaCl did not affect the acidification although it affected the VFA composition, promoting butyric acid concentration.

Versatile production of PHAs with mixed cultures and different substrates

A microbial mixed culture (MMC) was enriched with a mixture of VFAs (1.52:0.61:0.15:0.25 g/L of HAc:HPr:HBu:HVa) so as to achieve a community able to accumulate independently of the substrate so as to be able to tailor biopolymers.

The enrichment was successful and lead to a MMC able to achieve high biopolymer yields (0.683 - 0.838 Cmol PHA/Cmol VFA) and biopolymer production rates (0.184 - 0.338 Cmol PHA/(Cmol X · h)).

The biopolymers had different compositions depending on the substrate that ranged from values of HB:HV of 94:2 to 24:76 (wt% of PHA), with maximum accumulating values of 39.0 - 48.4 wt% PHA.

F/M ratios between approximately 1 and 7 Cmol VFA/(Cmol X · cycle) lead to optimal yields since lower values than 1 Cmol VFA/(Cmol X · cycle) indicate carbon

source limitation and higher values than 7 Cmol VFA/(Cmol X · cycle) the appearance of substrate inhibition.

The MMC was dominated by *Azoarcus* (~50%), *Comamonas* (~30%) and *Thauera* (~20%), which are related to high HV contents.

Transient concentrations of NaCl affect PHA accumulation in MMCs

An enriched MMC was used for the accumulation of biopolymers under transient conditions of NaCl.

Respirometric assays were performed previous before doing any accumulation assay. An IC_{50} of approximately 5 g NaCl/L was found. A inhibition of about the 85% of the biomass activity was found at 13 g NaCl/L. Batch assays were performed at 0, 7, 13 and 20 g NaCl/L so as to assess the observations done with the respirometric assays.

The maximum PHA inside the cells was affected by the NaCl concentration. The maximum value (53%) was achieved when no NaCl was present and it decreased to 9% when 20 g NaCl/L were present.

A very interesting observation is that the HB:HV ratio increased as the concentration of inhibitor also increased since the HB yield was not affected by the presence of NaCl but HV yield was. This was due to the decrease of HV in the biopolymer at higher NaCl concentrations since HPr is likely used for maintenance rather than for biopolymer production in comparison with other acids present.

PHA properties and its affectation by the purification stage or he presence of NaCl

PHA monomer composition determines at a great extent the biopolymer properties. In previous chapters, it was observed that the monomer composition can be modified by changes in the substrate but also by the presence of inhibitors like NaCl. It is also studied the influence of the extraction and purification stage.

Regarding the use of different substrates, different biopolymer compositions were achieved, with HV values ranging from 4 wt% to 66 wt% of PHA. It was

observed that the biopolymer properties are highly dependent on the HB content, with the exception of the degradation temperature (T_d).

Regarding the medium, the presence of NaCl in the reactor at a constant concentration of 21.6 g/L affected the maximum biopolymer accumulation, as expected from the results of Chapter 6. Salinity also affected the HV content in the cells by reducing it. Since the HB content was promoted, the thermal properties of the biopolymer were affected, but just in terms of composition. Nevertheless, the average molecular weight (M_w) was affected in a higher extent since it was not only dependent of the HB content. The variation in the M_w is a possible result of the fact that the shorter chains were consumed while higher chains were produced, as a presumable consequence of the inhibitory effect of the NaCl.

Finally, purification affected the thermal properties but it did not affect the M_w. Glass transition temperature (T_g), melting temperature (T_m) and Td decreased possibly by the fact that impurities can act as plasticisers.

PHA production using organic acids obtained from fish-canning wastewater

A MMC was enriched using a fermented fish-canning wastewater diluted with tap water. This gave a substrate composition of approximately 28.8:16.4:47.8:6.1 COD% of HAc:HPr:HBu:HVa, respectively; an ammonium content of 228.9 ± 47.7 mg NH_4^+-N/L; and a salinity of 3.19 ± 0.79 g NaCl/L. For testing the accumulation capacity and the influence of the feeding regime, two different accumulations were performed: feeding in pulses (fed-batch) and one single pulse feeding (batch).

Higher accumulation values were obtained in the fed-batch (8.35 wt%) in comparison with the batch (5.70 wt%). It can seem that pulse wise feeding is the optimum strategy but in terms of productivity values of 55.4 mg PHA/(L · h) were obtained in the batch assay, which is substantially higher than the value of 10.31 mg PHA/(L · h) obtained in the fed-batch assay.

Nevertheless, low accumulating rates were achieved since NaCl concentrations of 8 g NaCl/L were achieved. One indicator of this inhibition is the low HV production since propionic is used for maintenance under inhibitory conditions, as seen in Chapter 6. Another reason for the low kinetic and stoichiometric values for PHA

production can be attributed to the presence of high concentrations of NH_4^+, with yields for biomass growth of 0.366 and 0.266 Cmol X/Cmol VFA for the fed-batch and batch assays, respectively.

In global terms, results must be optimised to obtain a better enrichment which will subsequently improve the accumulation.

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